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(54) Title: ALZHEIMER'S DISEASE SECRETASE (54) Titre: SECRETASE DE LA MALADIE D'ALZHEIMER (57) Abstract <p>The present invention provides the enzyme and enzymatic procedures for cleaving the 'beta' secretase cleavage site of the APP protein and associated nucleic acids, peptides, vectors, cells and cell isolates and assays.</p> (57) Abrégé <p>La présente invention porte sur l'enzyme et les procédures enzymatiques de clivage du site de clivage de la 'beta' sécrétase de la protéine APP et des acides nucléiques, des peptides, des vecteurs, des cellules et des isolats cellulaires associés, et sur des dosages.</p>	

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<p>(21) International Application Number: PCT/US99/20881 (22) International Filing Date: 23 September 1999 (23.09.99) (30) Priority Data: 60/101,594 24 September 1998 (24.09.98) US (71) Applicant (for all designated States except US): PHARMACIA & UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GURNEY, Mark, E. [US/US]; 910 Rosewood Avenue, S.E., Grand Rapids, MI 49506 (US). BIENKOWSKI, Michael, Jerome [US/US]; 3431 Hollow Wood, Portage, MI 49024 (US). HEINRIK- SON, Robert, Leroy [US/US]; 81 South Lake Doster Drive, Plainwell, MI 49080 (US). PARODI, Luis, A. [US/SE]; Grevgafan 24, S-115 43 Stockholm (SE). YAN, Riqiang [US/US]; 5026 Queen Victoria Street, Kalamazoo, MI 49009 (US).</p>	<p>(74) Agent: WOOTTON, Thomas, A.; Pharmacia & Upjohn Com- pany, Intellectual Property Legal Services, 301 Henrietta Street, Kalamazoo, MI 49001 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published Without international search report and to be republished upon receipt of that report.</p>	
<p>(54) Title: ALZHEIMER'S DISEASE SECRETASE (57) Abstract The present invention provides the enzyme and enzymatic procedures for cleaving the β secretase cleavage site of the APP protein and associated nucleic acids, peptides, vectors, cells and cell isolates and assays.</p>		

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Description

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Alzheimer's Disease Secretase

FIELD OF THE INVENTION

The present invention related to the field of Alzheimer's Disease, APP, amyloid beta peptide, and human aspartyl proteases as well as a method for the identification of agents that modulate the activity of these polypeptides.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) causes progressive dementia with consequent formation of amyloid plaques, neurofibrillary tangles, gliosis and neuronal loss. The disease occurs in both genetic and sporadic forms whose clinical course and pathological features are quite similar. Three genes have been discovered to date which when mutated cause an autosomal dominant form of Alzheimer's disease. These encode the amyloid protein precursor (APP) and two related proteins, presenilin-1 (PS1) and presenilin-2 (PS2), which as their names suggest are both structurally and functionally related. Mutations in any of the three enhance proteolytic processing of APP via an intracellular pathway that produces amyloid beta peptide or the A β peptide (or sometimes here as Abeta), a 40-42 amino acid long peptide that is the primary component of amyloid plaque in AD. Dysregulation of intracellular pathways for proteolytic processing may be central to the pathophysiology of AD. In the case of plaque formation, mutations in APP, PS1 or PS2 consistently alter the proteolytic processing of APP so as to enhance formation of A β 1-42, a form of the A β peptide which seems to be particularly amyloidogenic, and thus very important in AD. Different forms of APP range in size from 695-770 amino acids, localize to the cell surface, and have a single C-terminal transmembrane domain. The Abeta peptide is derived from a region of APP adjacent to and containing a portion of the transmembrane domain. Normally, processing of APP at the α -secretase site cleaves the midregion of the A β sequence adjacent to the membrane and releases the soluble, extracellular domain of APP from the cell surface. This α -secretase APP processing, creates soluble APP- α , and it is normal and not thought to contribute to AD.

Pathological processing of APP at the β - and γ -secretase sites produces a very different result than processing at the α site. Sequential processing at the β - and γ -secretase sites releases the A β peptide, a peptide possibly very important in AD pathogenesis. Processing at the β - and γ -secretase sites can occur in both the endoplasmic reticulum (in neurons) and in the endosomal/lysosomal pathway after reinternalization of cell surface

5 APP (in all cells). Despite intense efforts, for 10 years or more, to identify the enzymes
responsible for processing APP at the β and γ sites, to produce the A β peptide, those
proteases remained unknown until this disclosure. Here, for the first time, we report the
10 identification and characterization of the β secretase enzyme. We disclose some known and
5 some novel human aspartic proteases that can act as β -secretase proteases and, for the first
time, we explain the role these proteases have in AD. We describe regions in the proteases
critical for their unique function and for the first time characterize their substrate. This is
15 the first description of expressed isolated purified active protein of this type, assays that use
the protein, in addition to the identification and creation of useful cell lines and inhibitors.

10 SUMMARY OF THE INVENTION

Here we disclose a number of variants of the asp2 gene and peptide.

20 Any isolated or purified nucleic acid polynucleotide that codes for a protease
capable of cleaving the beta (β) secretase cleavage site of APP that contains two or more
sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids
25 that code for about 100 to 300 amino acid positions, where the amino acids in those
positions may be any amino acids, where the first set of special nucleic acids consists of the
nucleic acids that code for the peptide DTG, where the first nucleic acid of the first special
set of nucleic acids is, the first special nucleic acid, and where the second set of nucleic
30 acids code for either the peptide DSG or DTG, where the last nucleic acid of the second set
of nucleic acids is the last special nucleic acid, with the proviso that the nucleic acids
disclosed in SEQ ID NO. 1 and SEQ. ID NO. 5 are not included. The nucleic acid
35 polynucleotide of claim 1 where the two sets of nucleic acids are separated by nucleic acids
that code for about 125 to 222 amino acid positions, which may be any amino acids. The
nucleic acid polynucleotide of claim 2 that code for about 150 to 172 amino acid positions,
25 which may be any amino acids. The nucleic acid polynucleotide of claim that code for
about 172 amino acid positions, which may be any amino acids. The nucleic acid
40 polynucleotide of claim 4 where the nucleotides are described in SEQ. ID. NO. 3 The
nucleic acid polynucleotide of claim 2 where the two sets of nucleic acids are separated by
nucleic acids that code for about 150 to 196 amino acid positions. The nucleic acid
45 polynucleotide of claim 6 where the two sets of nucleotides are separated by nucleic acids
that code for about 196 amino acids (positions). The nucleic acid polynucleotide of claim 7
where the two sets of nucleic acids are separated by the same nucleic acid sequences that
50 separate the same set of special nucleic acids in SEQ. ID. NO. 5. The nucleic acid

polynucleotide of claim 4 where the two sets of nucleic acids are separated by nucleic acids that code for about 150 to 190, amino acid (positions). The nucleic acid polynucleotide of claim 9 where the two sets of nucleotides are separated by nucleic acids that code for about 190 amino acids (positions). The nucleic acid polynucleotide of claim 10 where the two sets of nucleotides are separated by the same nucleic acid sequences that separate the same set of special nucleotides in SEQ. ID. NO. 1. Claims 1-11 where the first nucleic acid of the first special set of amino acids, that is, the first special nucleic acid, is operably linked to any codon where the nucleic acids of that codon codes for any peptide comprising from 1 to 10,000 amino acid (positions). The nucleic acid polynucleotide of claims 1-12 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any any reporter proteins or proteins which facilitate purification. The nucleic acid polynucleotide of claims 1-13 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin. Claims 1-14 where the last nucleic acid of the second set of special amino acids, that is, the last special nucleic acid, is operably linked to nucleic acid polymers that code for any peptide comprising any amino acids from 1 to 10,000 amino acids. Claims 1-15 where the last special nucleic acid is operably linked to any codon linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification. The nucleic acid polynucleotide of claims 1-16 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.

Any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code for DTG, where the first nucleic acid of the first special set of nucleic acids is, the first special nucleic acid, and where the second set of nucleic acids code for either DSG or DTG, where the last nucleic acid of the second set of special nucleic acids is the last special nucleic acid, where the first special nucleic acid is operably linked to nucleic

5 acids that code for any number of amino acids from zero to 81 amino acids and where each
of those codons may code for any amino acid. The nucleic acid polynucleotide of claim 18
10 , where the first special nucleic acid is operably linked to nucleic acids that code for any
number of from 64 to 77 amino acids where each codon may code for any amino acid. The
5 nucleic acid polynucleotide of claim 19 , where the first special nucleic acid is operably
linked to nucleic acids that code for 71 amino acids. The nucleic acid polynucleotide of
claim 20, where the first special nucleic acid is operably linked to 71 amino acids and
15 where the first of those 71 amino acids is the amino acid T. The nucleic acid
polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least
10 95% identical to SEQ. ID. (Example 11). The nucleic acid polynucleotide of claim 22,
where the complete polynucleotide comprises SEQ. ID. (Example 11). The nucleic acid
20 polynucleotide of claim 18 , where the first special nucleic acid is operably linked to nucleic
acids that code for any number of from 40 to 54 amino acids where each codon may code
for any amino acid. The nucleic acid polynucleotide of claim 24, where the first special
25 nucleic acid is operably linked to nucleic acids that code for 47 amino acids. The nucleic
acid polynucleotide of claim 20, where the first special nucleic acid is operably linked to 47
codons where the first those 47 amino acids is the amino acid E. The nucleic acid
polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least
30 95% identical to SEQ. ID. (Example 10). The nucleic acid polynucleotide of claim 22,
20 where the complete polynucleotide comprises SEQ. ID. (Example 10).

Any isolated or purified nucleic acid polynucleotide that codes for a protease
35 capable of cleaving the beta (β) secretase cleavage site of APP that contains two or more
sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids
that code for about 100 to 300 amino acid positions, where the amino acids in those
25 positions may be any amino acids, where the first set of special nucleic acids consists of the
40 nucleic acids that code for the peptide DTG, where the first nucleic acid of the first special
set of amino acids is, the first special nucleic acid, and where the second set of special
nucleic acids code for either the peptide DSG or DTG, where the last nucleic acid of the
45 second set of special nucleic acids, the last special nucleic acid, is operably linked to
30 nucleic acids that code for any number of codons from 50 to 170 codons. The nucleic acid
polynucleotide of claim 29 where the last special nucleic acid is operably linked to nucleic
acids comprising from 100 to 170 codons. The nucleic acid polynucleotide of claim 30
50 where the last special nucleic acid is operably linked to nucleic acids comprising from 142

5 to 163 codons. The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 142 codons. The nucleic acid polynucleotide of claim 32 where the polynucleotide comprises a sequence that is at least 95% identical to SEQ. ID. (Example 9 or 10). The nucleic acid polynucleotide of claim 10 5 33, where the complete polynucleotide comprises SEQ. ID. (Example 9 or 10). The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 163 codons. The nucleic acid polynucleotide of claim 35 where the polynucleotide comprises a sequence that is at least 95% identical to SEQ. ID. (Example 9 or 10). The nucleic acid polynucleotide of claim 36, where the 15 10 complete polynucleotide comprises SEQ. ID. (Example 9 or 10). The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 170 codons. Claims 1-38 where the second set of special nucleic acids code for the peptide DSG, and optionally the first set of nucleic acid polynucleotide is operably linked to a peptide purification tag. Claims 1-39 where the nucleic acid 20 15 polynucleotide is operably linked to a peptide purification tag which is six histidine. Claims 1-40 where the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a second polynucleotide, where both first and second polynucleotides have at least 50 codons. Claims 1-40 where the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a 30 20 second polynucleotide, where both first and second polynucleotides have at least 50 codons where both said polynucleotides are in the same solution. A vector which contains a polynucleotide described in claims 1-42. A cell or cell line which contains a polynucleotide described in claims 1-42.

Any isolated or purified peptide or protein comprising an amino acid polymer that is 25 a protease capable of cleaving the beta (β) secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 40 100 to 300 amino acid positions, where each amino acid position can be any amino acid, where the first set of special amino acids consists of the peptide DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, where the second 45 30 set of amino acids is selected from the peptide comprising either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, with the proviso that the proteases disclosed in SEQ ID NO. 2 and SEQ. ID NO. 6 are not 50 included. The amino acid polypeptide of claim 45 where the two sets of amino acids are

5 separated by about 125 to 222 amino acid positions where in each position it may be any amino acid. The amino acid polypeptide of claim 46 where the two sets of amino acids are separated by about 150 to 172 amino acids. The amino acid polypeptide of claim 47 where the two sets of amino acids are separated by about 172 amino acids. The amino acid
10 5 polypeptide of claim 48 where the protease is described in SEQ. ID. NO. 4 The amino acid polypeptide of claim 46 where the two sets of amino acids are separated by about 150 to 196 amino acids. The amino acid polypeptide of claim 50 where the two sets of amino acids are separated by about 196 amino acids. The amino acid polypeptide of claim 51
15 where the two sets of amino acids are separated by the same amino acid sequences that separate the same set of special amino acids in SEQ. ID. NO. 6. The amino acid polypeptide of claim 46 where the two sets of amino acids are separated by about 150 to 190, amino acids. The amino acid polypeptide of claim 53 where the two sets of
20 nucleotides are separated by about 190 amino acids. The amino acid polypeptide of claim 54 where the two sets of nucleotides are separated by the same amino acid sequences that separate the same set of special amino acids in SEQ. ID. NO. 2. Claims 45-55 where the first amino acid of the first special set of amino acids, that is, the first special amino acid, is operably linked to any peptide comprising from 1 to 10,000 amino acids. The amino acid polypeptide of claims 45-56 where the first special amino acid is operably linked to any
25 peptide selected from the group consisting of: any any reporter proteins or proteins which facilitate purification. The amino acid polypeptide of claims 45-57 where the first special amino acid is operably linked to any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green
30 Fluorescent protein, and ubiquitin. Claims 45-58, where the last amino acid of the second set of special amino acids, that is, the last special amino acid, is operably linked to any peptide comprising any amino acids from 1 to 10,000 amino acids. Claims 45-59 where the last special amino acid is operably linked any peptide selected from the group consisting of
35 any reporter proteins or proteins which facilitate purification. The amino acid polypeptide of claims 45-60 where the first special amino acid is operably linked to any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.
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Any isolated or purified peptide or protein comprising an amino acid polypeptide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that
50 contains two or more sets of special amino acids, where the special amino acids are

5 separated by about 100 to 300 amino acid positions, where each amino acid in each position
can be any amino acid, where the first set of special amino acids consists of the amino acids
DTG, where the first amino acid of the first special set of amino acids is, the first special
10 amino acid, D, and where the second set of amino acids is either DSG or DTG, where the
last amino acid of the second set of special amino acids is the last special amino acid, G,
where the first special amino acid is operably linked to amino acids that code for any
number of amino acids from zero to 81 amino acid positions where in each position it may
15 be any amino acid. The amino acid polypeptide of claim 62, where the first special amino
acid is operably linked to a peptide from about 64 to 77 amino acids positions where each
amino acid position may be any amino acid. The amino acid polypeptide of claim 63,
where the first special amino acid is operably linked to a peptide of 71 amino acids. The
20 amino acid polypeptide of claim 64, where the first special amino acid is operably linked to
71 amino acids and the first of those 71 amino acids is the amino acid T. The amino acid
polypeptide of claim 65, where the polypeptide comprises a sequence that is at least 95%
15 identical to SEQ. ID. (Example 11). The amino acid polypeptide of claim 66, where the
complete polypeptide comprises SEQ. ID. (Example 11). The amino acid polypeptide of
claim 62, where the first special amino acid is operably linked to any number of from 40 to
54 amino acids (positions) where each amino acid position may be any amino acid. The
30 amino acid polypeptide of claim 68, where the first special amino acid is operably linked to
amino acids that code for a peptide of 47 amino acids. The amino acid polypeptide of claim
69, where the first special amino acid is operably linked to a 47 amino acid peptide where
the first those 47 amino acids is the amino acid E. The amino acid polypeptide of claim 70,
35 where the polypeptide comprises a sequence that is at least 95% identical to SEQ. ID.
(Example 10). The amino acid polypeptide where the polypeptide comprises Example 10).

25 Any isolated or purified amino acid polypeptide that is a protease capable of
cleaving the beta (β) secretase cleavage site of APP that contains two or more sets of
40 special amino acids, where the special amino acids are separated by about 100 to 300 amino
acid positions, where each amino acid in each position can be any amino acid, where the
first set of special amino acids consists of the amino acids that code for DTG, where the
45 first amino acid of the first special set of amino acids is, the first special amino acid, D, and
30 where the second set of amino acids are either DSG or DTG, where the last amino acid of
the second set of special amino acids is the last special amino acid, G, which is operably
50 linked to any number of amino acids from 50 to 170 amino acids, which may be any amino

acids. The amino acid polypeptide of claim 73 where the last special amino acid is operably linked to a peptide of about 100 to 170 amino acids. The amino acid polypeptide of claim 74 where the last special amino acid is operably linked to a peptide of about 142 to 163 amino acids. The amino acid polypeptide of claim 75 where the last special amino acid is operably linked to a peptide of about 142 amino acids. The amino acid polypeptide of claim 76 where the polypeptide comprises a sequence that is at least 95% identical to SEQ. ID. (Example 9 or 10). The amino acid polypeptide of claim 75 where the last special amino acid is operably linked to a peptide of about 163 amino acids. The amino acid polypeptide of claim 79 where the polypeptide comprises a sequence that is at least 95% identical to SEQ. ID. (Example 9 or 10). The amino acid polypeptide of claim 79, where the complete polypeptide comprises SEQ. ID. (Example 9 or 10). The amino acid polypeptide of claim 74 where the last special amino acid is operably linked to a peptide of about 170 amino acids. Claim 46-81 where the second set of special amino acids is comprised of the peptide with the amino acid sequence DSG. Claims 45-82 where the amino acid polypeptide is operably linked to a peptide purification tag. Claims 45-83 where the amino acid polypeptide is operably linked to a peptide purification tag which is six histidine. Claims 45-84 where the first set of special amino acids are on one polypeptide and the second set of special amino acids are on a second polypeptide, where both first and second polypeptide have at least 50 amino acids, which may be any amino acids. Claims 45-84 where the first set of special amino acids are on one polypeptide and the second set of special amino acids are on a second polypeptide, where both first and second polypeptides have at least 50 amino acids where both said polypeptides are in the same vessel. A vector which contains a polypeptide described in claims 45-86. A cell or cell line which contains a polynucleotide described in claims 45-87. The process of making any of the polynucleotides, vectors, or cells of claims 1-44. The process of making any of the polypeptides, vectors or cells of claims 45-88. Any of the polynucleotides, polypeptides, vectors, cells or cell lines described in claims 1-88 made from the processes described in claims 89 and 90.

Any isolated or purified peptide or protein comprising an amino acid polypeptide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 100 to 300 amino acid positions, where each amino acid in each position can be any amino acid, where the first set of special amino acids consists of the amino acids

5 DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, D, and where the second set of amino acids is either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, G, where the first special amino acid is operably linked to amino acids that code for any
10 5 number of amino acids from zero to 81 amino acid positions where in each position it may be any amino acid.

The amino acid polypeptide of claim 62, where the first special amino acid is operably linked to a peptide from about 30 to 77 amino acids positions where each amino acid position may be any amino acid. The amino acid polypeptide of claim 63, where the
15 10 first special amino acid is operably linked to a peptide of 35, 47, 71, or 77 amino acids.

The amino acid polypeptide of claim 63, where the first special amino acid is operably linked to the same corresponding peptides from SEQ. ID. NO. 3 that are 35, 47, 71, or 77 peptides in length, beginning counting with the amino acids on the first special sequence, DTG, towards the N-terminal of SEQ. ID. NO. 3.
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15 The amino acid polypeptide of claim 65, where the polypeptide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ. ID. NO. 4, that is, identical to that portion of the sequences in SEQ. ID. NO. 4, including all the sequences from both the first and or the second special nucleic acids, toward the N-terminal, through and including 71, 47, 35 amino acids before the first special amino acids.
25 30 (Examples 10 and 11).

The amino acid polypeptide of claim 65, where the complete polypeptide comprises the peptide of 71 amino acids, where the first of the amino acid is T and the second is Q. The nucleic acid polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ. ID.
35 25 NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 71 amino acids, see Example 10, beginning from the DTG site and including the nucleotides from that code for 71 amino acids).
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45 The nucleic acid polynucleotide of claim 22, where the complete polynucleotide
30 comprises identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 71
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amino acids, see Example 10, beginning from the DTG site and including the nucleotides from that code for 71 amino acids).

The nucleic acid polynucleotide of claim 18, where the first special nucleic acid is operably linked to nucleic acids that code for any number of from about 30 to 54 amino acids where each codon may code for any amino acid.

The nucleic acid polynucleotide of claim 20, where the first special nucleic acid is operably linked to 47 codons where the first those 35 or 47 amino acids is the amino acid E or G.

The nucleic acid polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to that portion of the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 35 or 47 amino acids, see Example 11 for the 47 example, beginning from the DTG site and including the nucleotides from that code for the previous 35 or 47 amino acids before the DTG site). The nucleic acid polynucleotide of claim 22, where the polynucleotide comprises identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 35 or 47 amino acids, see Example 11 for the 47 example, beginning from the DTG site and including the nucleotides from that code for the previous 35 or 47 amino acids before the DTG site).

An isolated nucleic acid molecule comprising a polynucleotide, said polynucleotide encoding a Hu-Asp polypeptide and having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), wherein said Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) polypeptides have the complete amino acid sequence of SEQ ID No. 2, SEQ ID No. 4, and SEQ ID No. 6, respectively; and

(b) a nucleotide sequence complementary to the nucleotide sequence of (a).

The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is Hu-Asp1, and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID No. 1. The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is Hu-

5 Asp2(a), and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of
SEQ ID No. 4. The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is
Hu-Asp2(b), and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of
10 SEQ ID No. 5. An isolated nucleic acid molecule comprising polynucleotide which
hybridizes under stringent conditions to a polynucleotide having the nucleotide sequence in
(a) or (b) of claim 92. A vector comprising the nucleic acid molecule of claim 96. The
vector of claim 97, wherein said nucleic acid molecule is operably linked to a promoter for
the expression of a Hu-Asp polypeptide. The vector of claim 98, wherein said Hu-Asp
15 polypeptide is Hu-Asp1. The vector of claim 98, wherein said Hu-Asp polypeptide is Hu-
Asp2(a). The vector of claim 98, wherein said Hu-Asp polypeptide is Hu-Asp2(b). A host
cell comprising the vector of claim 98. A method of obtaining a Hu-Asp polypeptide
comprising culturing the host cell of claim 102 and isolating said Hu-Asp polypeptide. An
isolated Hu-Asp1 polypeptide comprising an amino acid sequence at least 95% identical to
20 a sequence comprising the amino acid sequence of SEQ ID No. 2. An isolated Hu-Asp2(a)
polypeptide comprising an amino acid sequence at least 95% identical to a sequence
comprising the amino acid sequence of SEQ ID No. 4. An isolated Hu-Asp2(a) polypeptide
comprising an amino acid sequence at least 95% identical to a sequence comprising the
amino acid sequence of SEQ ID No. 8. An isolated antibody that binds specifically to the
Hu-Asp polypeptide of any of claims 104-107.

20 Here we disclose numerous methods to assay the enzyme.

A method to identify a cell that can be used to screen for inhibitors of β
secretase activity comprising:

35 (a) identifying a cell that expresses a protease capable of cleaving APP at the β
secretase site, comprising:

- 25 i) collect the cells or the supernatant from the cells to be identified
- 40 ii) measure the production of a critical peptide, where the critical
peptide is selected from the group consisting of either the APP C-
terminal peptide or soluble APP,
- 45 iii) select the cells which produce the critical peptide.

30 The method of claim 108 where the cells are collected and the critical peptide is the
APP C-terminal peptide created as a result of the β secretase cleavage. The method of claim
108 where the supernatant is collected and the critical peptide is soluble APP where the
soluble APP has a C-terminal created by β secretase cleavage. The method of claim 108

5 where the cells contain any of the nucleic acids or polypeptides of claims 1-86 and where
the cells are shown to cleave the β secretase site of any peptide having the following
peptide structure, P2, P1, P1', P2', where P2 is K or N, where P1 is M or L, where P1' is
D, where P2' is A. The method of claim 111 where P2 is K and P1 is M.. The method of
10 5 claim 112 where P2 is N and P1 is L.

Any bacterial cell comprising any nucleic acids or peptides in claims 1-86
and 92-107. A bacterial cell of claim 114 where the bacteria is *E coli*. Any eukaryotic cell
comprising any nucleic acids or polypeptides in claims 1-86 and 92-107.

15 Any insect cell comprising any of the nucleic acids or polypeptides in claims
10 1-86 and 92-107. A insect cell of claim 117 where the insect is sf9, or High 5. A insect
cell of claim 100 where the insect cell is High 5. A mammalian cell comprising any of the
20 nucleic acids or polypeptides in claims 1-86 and 92-107. A mammalian cell of claim 120
where the mammalian cell is selected from the group consisting of, human, rodent,
lagomorph, and primate. A mammalian cell of claim 121 where the mammalian cell is
15 selected from the group consisting of human cell. A mammalian cell of claim 122 where
the human cell is selected from the group comprising HEK293, and IMR-32. A
25 mammalian cell of claim 121 where the cell is a primate cell. A primate cell of claim 124
where the primate cell is a COS-7 cell. A mammalian cell of claim 121 where cell is
30 selected from a rodent cells. A rodent cell of claim 126 selected from, CHO-K1, Neuro-
20 2A, 3T3 cells. A yeast cell of claim 115. An avian cell of claim 115.

Any isoform of APP where the last two carboxy terminus amino acids of that
isoform are both lysine residues. In written descrip. Define isoform is any APP
35 polypeptide, including APP variants (including mutations), and APP fragments that exists
in humans such as those described in US 5,766,846, col 7, lines 45-67, incorporated into this
25 document by reference. The isoform of APP from claim 114, comprising the isoform
40 known as APP695 modified so that its last two having two lysine residues as its last two
carboxy terminus amino acids. The isoform of claim 130 comprising SEQ. ID. 16. The
isoform variant of claim 130 comprising SEQ. ID. NO. 18, and 20. Any eukaryotic cell
45 line, comprising nucleic acids or polypeptides of claim 130-132. Any cell line of claim 133
30 that is a mammalian cell line (HEK293, Neuro2a, best - plus others. A method for
identifying inhibitors of an enzyme that cleaves the beta secretase cleavable site of APP
comprising:

5 a) culturing cells in a culture medium under conditions in which the enzyme causes processing of APP and release of amyloid beta-peptide into the medium and causes the accumulation of CTF99 fragments of APP in cell lysates.

10 b) exposing the cultured cells to a test compound; and specifically determining whether the test compound inhibits the function of the enzyme by measuring the amount of amyloid beta-peptide released into the medium and or the amount of CTF99 fragments of APP in cell lysates;

15 c) identifying test compounds diminishing the amount of soluble amyloid beta peptide present in the culture medium and diminution of CTF99 fragments of APP in cell lysates as Asp2 inhibitors.

20 The method of claim 135 wherein the cultured cells are a human, rodent or insect cell line. The method of claim 136 wherein the human or rodent cell line exhibits β secretase activity in which processing of APP occurs with release of amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates. A method as in claim 25 137 wherein the human or rodent cell line treated with the antisense oligomers directed against the enzyme that exhibits β secretase activity, reduces release of soluble amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates. A method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method 30 comprising:

35 a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

25 b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

40 whereby a lower level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has decreased the activity of said Hu-Asp polypeptide. The nucleic acids, peptides, proteins, vectors, cells and cell lines, and assays described herein.

45 30 The present invention provides isolated nucleic acid molecules comprising a polynucleotide that codes for a polypeptide selected from the group consisting of human aspartyl proteases. In particular, human aspartyl protease 1 (Hu-Asp1) and two alternative 50 splice variants of human aspartyl protease 2 (Hu-Asp2), designated herein as Hu-Asp2(a) and

5 Hu-Asp2(b). As used herein, all references to "Hu-Asp" should be understood to refer to all of
Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b). In addition, as used herein, all references to "Hu-
Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). Hu-Asp1 is
expressed most abundantly in pancreas and prostate tissues, while Hu-Asp2(a) and Hu-
10 Asp2(b) are expressed most abundantly in pancreas and brain tissues. The invention also
provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments
thereof which exhibit aspartyl protease activity.

15 In a preferred embodiment, the nucleic acid molecules comprise a polynucleotide
having a nucleotide sequence selected from the group consisting of residues 1-1554 of SEQ
10 ID NO:1, encoding Hu-Asp1, residues 1-1503 of SEQ ID NO:3, encoding Hu-Asp2(a), and
residues 1-1428 of SEQ ID NO:5, encoding Hu-Asp2(b). In another aspect, the invention
provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes
20 under stringent conditions to a polynucleotide encoding Hu-Asp1, Hu-Asp2(a), Hu-Asp2(b),
or fragments thereof. European patent application EP 0 848 062 discloses a polypeptide
referred to as "Asp 1," that bears substantial homology to Hu-Asp1, while international
25 application WO 98/22597 discloses a polypeptide referred to as "Asp 2," that bears substantial
homology to Hu-Asp2(a).

30 The present invention also provides vectors comprising the isolated nucleic acid
molecules of the invention, host cells into which such vectors have been introduced, and
20 recombinant methods of obtaining a Hu-Asp1, Hu-Asp2(a), or Hu-Asp2(b) polypeptide
comprising culturing the above-described host cell and isolating the relevant polypeptide.

35 In another aspect, the invention provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-
Asp2(b) polypeptides, as well as fragments thereof. In a preferred embodiment, the Hu-Asp1,
Hu-Asp2(a), and Hu-Asp2(b) polypeptides have the amino acid sequence given in SEQ ID
25 NO:2, SEQ ID NO:4, or SEQ ID NO:6, respectively. The present invention also describes
active forms of Hu-Asp2, methods for preparing such active forms, methods for preparing
40 soluble forms, methods for measuring Hu-Asp2 activity, and substrates for Hu-Asp2 cleavage.
The invention also describes antisense oligomers targeting the Hu-Asp1, Hu-Asp2(a) and Hu-
Asp2(b) mRNA transcripts and the use of such antisense reagents to decrease such mRNA
45 and consequently the production of the corresponding polypeptide. Isolated antibodies, both
30 polyclonal and monoclonal, that binds specifically to any of the Hu-Asp1, Hu-Asp2(a), and
Hu-Asp2(b) polypeptides of the invention are also provided.

The invention also provides a method for the identification of an agent that modulates the activity of any of Hu-Asp-1, Hu-Asp2(a), and Hu-Asp2(b). The invention describes methods to test such agents in cell-free assays to which Hu-Asp2 polypeptide is added, as well as methods to test such agents in human or other mammalian cells in which Hu-Asp2 is present.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

Sequence ID No. 1—Human Asp-1, nucleotide sequence
 Sequence ID No. 2—Human Asp-1, predicted amino acid sequence
 Sequence ID No. 3—Human Asp-2(a), nucleotide sequence
 Sequence ID No. 4—Human Asp-2(a), predicted amino acid sequence
 Sequence ID No. 5—Human Asp-2(b), nucleotide sequence
 Sequence ID No. 6—Human Asp-2(b), predicted amino acid sequence
 Sequence ID No. 7—Murine Asp-2(a), nucleotide sequence
 Sequence ID No. 8—Murine Asp-2(a), predicted amino acid sequence
 Sequence ID No. 9—Human APP695, nucleotide sequence
 Sequence ID No. 10—Human APP695, predicted amino acid sequence
 Sequence ID No. 11—Human APP695-Sw, nucleotide sequence
 Sequence ID No. 12—Human APP695-Sw, predicted amino acid sequence
 Sequence ID No. 13—Human APP695-VF, nucleotide sequence
 Sequence ID No. 14—Human APP695-VF, predicted amino acid sequence
 Sequence ID No. 15—Human APP695-KK, nucleotide sequence
 Sequence ID No. 16—Human APP695-KK, predicted amino acid sequence
 Sequence ID No. 17—Human APP695-Sw-KK, nucleotide sequence
 Sequence ID No. 18—Human APP695-Sw-KK, predicted amino acid sequence
 Sequence ID No. 19—Human APP695-VF-KK, nucleotide sequence
 Sequence ID No. 20—Human APP695-VF-KK, predicted amino acid sequence
 Sequence ID No. 21—T7-Human-pro-Asp-2(a) Δ TM, nucleotide sequence
 Sequence ID No. 22—T7-Human-pro-Asp-2(a) Δ TM, amino acid sequence
 Sequence ID No. 23—T7-Caspase-Human-pro-Asp-2(a) Δ TM, nucleotide sequence
 Sequence ID No. 24—T7-Caspase-Human-pro-Asp-2(a) Δ TM, amino acid sequence
 Sequence ID No. 25—Human-pro-Asp-2(a) Δ TM (low GC), nucleotide sequence
 Sequence ID No. 26—Human-pro-Asp-2(a) Δ TM, (low GC), amino acid sequence
 Sequence ID No. 27—T7-Caspase-Caspase 8 cleavage-Human-pro-Asp-2(a) Δ TM, nucleotide sequence
 Sequence ID No. 28—T7-Caspase-Caspase 8 cleavage-Human-pro-Asp-2(a) Δ TM, amino acid sequence
 Sequence ID No. 29—Human Asp-2(a) Δ TM, nucleotide sequence
 Sequence ID No. 30—Human Asp-2(a) Δ TM, amino acid sequence
 Sequence ID No. 31—Human Asp-2(a) Δ TM(His)₆, nucleotide sequence
 Sequence ID No. 32—Human Asp-2(a) Δ TM(His)₆, amino acid sequence
 Sequence ID No.s 33-46 are described below in the Detailed Description of the Invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Figure 1 shows the nucleotide (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human Asp1.

Figure 2: Figure 2 shows the nucleotide (SEQ ID NO:3) and predicted amino acid sequence (SEQ ID NO:4) of human Asp2(a).

Figure 3: Figure 3 shows the nucleotide (SEQ ID NO:5) and predicted amino acid sequence (SEQ ID NO:6) of human Asp2(b). The predicted transmembrane domain of Hu-Asp2(b) is enclosed in brackets.

Figure 4: Figure 4 shows the nucleotide (SEQ ID No. 7) and predicted amino acid sequence (SEQ ID No. 8) of murine Assp2(a)

Figure 5: Figure 5 shows the BestFit alignment of the predicted amino acid sequences of Hu-Asp2(a) and murine Asp2(a)

Figure 6: Figure 6 shows the nucleotide (SEQ ID No. 21) and predicted amino acid sequence (SEQ ID No. 22) of T7-Human-pro-Asp-2(a) Δ TM

Figure 7: Figure 7 shows the nucleotide (SEQ ID No. 23) and predicted amino acid sequence (SEQ ID No. 24) of T7-caspase-Human-pro-Asp-2(a) Δ TM

Figure 8: Figure 8 shows the nucleotide (SEQ ID No. 25) and predicted amino acid sequence (SEQ ID No. 26) of Human-pro-Asp-2(a) Δ TM (low GC)

Figure 9: Western blot showing reduction of CTF99 production by HEK125.3 cells transfected with antisense oligomers targeting the Hu-Asp2 Mma

Figure 10: Western blot showing increase in CTF99 production in mouse Neuro-2a cells cotransfected with APP-KK with and without Hu-Asp2 only in those cells cotransfected with Hu-Asp2. A further increase in CTF99 production is seen in cells cotransfected with APP-Sw-KK with and without Hu-Asp2 only in those cells cotransfected with Hu-Asp2

Figure 11: Figure 11 shows the predicted amino acid sequence (SEQ ID No. 30) of Human-Asp2(a) Δ TM

Figure 12: Figure 11 shows the predicted amino acid sequence (SEQ ID No. 30) of Human-Asp2(a) Δ TM(His)₆

DETAILED DESCRIPTION OF THE INVENTION

A few definitions used in this invention follow, most definitions to be used are those that would be used by one ordinarily skilled in the art.

When the β amyloid peptide any peptide resulting from beta secretase cleavage of APP. This includes, peptides of 39, 40, 41, 42 and 43 amino acids, extending from the β -

5 secretase cleavage site to 39, 40, 41, 42 and 43 amino acids. β amyloid peptide also means
sequences 1-6, SEQ. ID. NO. 1-6 of US 5,750,349, issued 12 May 1998 (incorporated into
this document by reference). A β -secretase cleavage fragment disclosed here is called CTF-
99, which extends from β -secretase cleavage site to the carboxy terminus of APP.

10 5 When an isoform of APP is discussed then what is meant is any APP polypeptide,
including APP variants (including mutations), and APP fragments that exists in humans
such as those described in US 5,766,846, col 7, lines 45-67, incorporated into this document
by reference and see below.

15 The term " β -amyloid precursor protein" (APP) as used herein is defined as a
10 polypeptide that is encoded by a gene of the same name localized in humans on the long
arm of chromosome 21 and that includes " β AP - here " β -amyloid protein" see above,
within its carboxyl third. APP is a glycosylated, single-membrane spanning protein
expressed in a wide variety of cells in many mammalian tissues. Examples of specific
isotypes of APP which are currently known to exist in humans are the 695-amino acid
25 polypeptide described by Kang et. al. (1987) Nature 325:733-736 which is designated as the
"normal" APP; the 751-amino acid polypeptide described by Ponte et al. (1988) Nature
331:525-527 (1988) and Tanzi et al. (1988) Nature 331:528-530; and the 770-amino acid
polypeptide described by Kitaguchi et. al. (1988) Nature 331:530-532. Examples of specific
30 variants of APP include point mutation which can differ in both position and phenotype (for
review of known variant mutation see Hardy (1992) Nature Genet. 1:233-234). All
references cited here incorporated by reference. The term "APP fragments" as used herein
refers to fragments of APP other than those which consist solely of β AP or β AP fragments.
35 That is, APP fragments will include amino acid sequences of APP in addition to those
which form intact 3AP or a fragment of β AP.

25 40 When the term "any amino acid" is used, the amino acids referred to are to be selected
from the following, three letter and single letter abbreviations - which may also be used, are
provided as follows:

45 Alanine, Ala, A; Arginine, Arg, R; Asparagine, Asn, N; Aspartic acid, Asp, D;
Cystein, Cys, C; Glutamine, Gln, Q; l-glutamic acid, Glu, E; Glycine, Gly, G;
30 Histidine, His, H; Isoleucine, Ile, I; Leucine, Leu, L; Lysine, Lys, K; Methionine, Met,
M; Phenylalanine, Phe, F; Proline, Pro, P; Serine, Ser, S; Threonine, Thr, T; Tryptophan,
Trp, W; Tyrosine, Tyr, Y; Valine, Val, V; Aspartic acid or Asparagine, Asx, B; Glutamic
50 acid or Glutamine, Glx, Z; Any amino acid, Xaa, X..

5 The present invention describes a method to scan gene databases for the simple
active site motif characteristic of aspartyl proteases. Eukaryotic aspartyl proteases such as
pepsin and renin possess a two-domain structure which folds to bring two aspartyl residues
into proximity within the active site. These are embedded in the short tripeptide motif
10 DTG, or more rarely, DSG. Most aspartyl proteases occur as proenzyme whose N-terminus
must be cleaved for activation. The DTG or DSG active site motif appears at about residue
65-70 in the proenzyme (prorenin, pepsinogen), but at about residue 25-30 in the active
enzyme after cleavage of the N-terminal prodomain. The limited length of the active site
15 motif makes it difficult to search collections of short, expressed sequence tags (EST) for
novel aspartyl proteases. EST sequences typically average 250 nucleotides or less, and so
would encode 80-90 amino acid residues or less. That would be too short a sequence to
span the two active site motifs. The preferred method is to scan databases of hypothetical
or assembled protein coding sequences. The present invention describes a computer
20 method to identify candidate aspartyl proteases in protein sequence databases. The method
was used to identify seven candidate aspartyl protease sequences in the *Caenorhabditis*
elegans genome. These sequences were then used to identify by homology search Hu-Asp1
and two alternative splice variants of Hu-Asp2, designated herein as Hu-Asp2(a) and Hu-
Asp2(b).

30 In a major aspect of the invention disclosed here we provide new information about
APP processing. Pathogenic processing of the amyloid precursor protein (APP) via the
A β pathway requires the sequential action of two proteases referred to as β -secretase and γ -
secretase. Cleavage of APP by the β -secretase and γ -secretase generates the N-terminus
35 and C-terminus of the A β peptide, respectively. Because over production of the A β
peptide, particularly the A β_{1-42} , has been implicated in the initiation of Alzheimer's disease,
25 inhibitors of either the β -secretase and/or the γ -secretase have potential in the treatment of
Alzheimer's disease. Despite the importance of the β -secretase and γ -secretase in the
pathogenic processing of APP, molecular definition of these enzymes has not been
accomplished to date. That is, it was not known what enzymes were required for cleavage
40 at either the β -secretase or the γ -secretase cleavage site. The sites themselves were
known because APP was known and the A β_{1-42} peptide was known, see US 5,766,846 and
US 5,837,672, (incorporated by reference, with the exception to reference to "soluble"
45 peptides). But what enzyme was involved in producing the A β_{1-42} peptide was unknown.

5 The present invention involves the molecular definition of several novel human
aspartyl proteases and one of these, referred to as Hu-Asp-2(a) and Hu-Asp2(b), has been
characterized in detail. Previous forms of asp1 and asp 2 have been disclosed, see EP
0848062 A2 and EP 0855444A2, inventors David Powel et. al., assigned to Smith Kline
10 Beecham Corp. (incorporated by reference). Herein are disclosed old and new forms of
Hu-Asp 2. For the first time they are expressed in active form, their substrates are
disclosed, and their specificity is disclosed. Prior to this disclosure cell or cell extracts were
required to cleave the β -secretase site, now purified protein can be used in assays, also
15 described here. Based on the results of (1) antisense knock out experiments, (2) transient
transfection knock in experiments, and (3) biochemical experiments using purified
recombinant Hu-Asp-2, we demonstrate that Hu-Asp-2 is the β -secretase involved in the
processing of APP. Although the nucleotide and predicted amino acid sequence of Hu-
20 Asp-2(a) has been reported, see above, see EP 0848062 A2 and EP 0855444A2, no
functional characterization of the enzyme was disclosed. Here the authors characterize the
Hu-Asp-2 enzyme and are able to explain why it is a critical and essential enzyme required
15 in the formation of $A\beta_{1-42}$, peptide and possible a critical step in the development of AD.

In another embodiment the present invention also describes a novel splice variant of
Hu-Asp2, referred to as Hu-Asp-2(b), that has never before been disclosed.

30 In another embodiment, the invention provides isolated nucleic acid molecules
comprising a polynucleotide encoding a polypeptide selected from the group consisting of
human aspartyl protease 1 (Hu-Asp1) and two alternative splice variants of human aspartyl
protease 2 (Hu-Asp2), designated herein as Hu-Asp2(a) and Hu-Asp2(b). As used herein, all
35 references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b).
Hu-Asp1 is expressed most abundantly in pancreas and prostate tissues, while Hu-Asp2(a)
and Hu-Asp2(b) are expressed most abundantly in pancreas and brain tissues. The invention
25 also provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as
fragments thereof which exhibit aspartyl protease activity.

The predicted amino acid sequences of Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) share
45 significant homology with previously identified mammalian aspartyl proteases such as
pepsinogen A, pepsinogen B, cathepsin D, cathepsin E, and renin. P.B.Szecs, *Scand. J. Clin.*
30 *Lab. Invest.* 52:(Suppl. 210 5-22 (1992)). These enzymes are characterized by the presence of
a duplicated DTG/DSG sequence motif. The Hu-Asp1 and HuAsp2 polypeptides disclosed

5 herein also exhibit extremely high homology with the ProSite consensus motif for aspartyl
proteases extracted from the SwissProt database.

10 The nucleotide sequence given as residues 1-1554 of SEQ ID NO:1 corresponds to
the nucleotide sequence encoding Hu-Asp1, the nucleotide sequence given as residues 1-1503
15 of SEQ ID NO:3 corresponds to the nucleotide sequence encoding Hu-Asp2(a), and the
nucleotide sequence given as residues 1-1428 of SEQ ID NO:5 corresponds to the nucleotide
sequence encoding Hu-Asp2(b). The isolation and sequencing of DNA encoding Hu-Asp1,
Hu-Asp2(a), and Hu-Asp2(b) is described below in Examples 1 and 2.

15 As is described in Examples 1 and 2, automated sequencing methods were used to
20 obtain the nucleotide sequence of Hu-Asp1, Hu-Asp2(a), and Hu-Asp-2(b). The Hu-Asp
nucleotide sequences of the present invention were obtained for both DNA strands, and are
believed to be 100% accurate. However, as is known in the art, nucleotide sequence obtained
by such automated methods may contain some errors. Nucleotide sequences determined by
25 automation are typically at least about 90%, more typically at least about 95% to at least about
99.9% identical to the actual nucleotide sequence of a given nucleic acid molecule. The
actual sequence may be more precisely determined using manual sequencing methods, which
are well known in the art. An error in sequence which results in an insertion or deletion of
one or more nucleotides may result in a frame shift in translation such that the predicted
30 amino acid sequence will differ from that which would be predicted from the actual
nucleotide sequence of the nucleic acid molecule, starting at the point of the mutation. The
Hu-Asp DNA of the present invention includes cDNA, chemically synthesized DNA, DNA
isolated by PCR, genomic DNA, and combinations thereof. Genomic Hu-Asp DNA may be
35 obtained by screening a genomic library with the Hu-Asp2 cDNA described herein, using
methods that are well known in the art, or with oligonucleotides chosen from the Hu-Asp2
sequence that will prime the polymerase chain reaction (PCR). RNA transcribed from Hu-
40 Asp DNA is also encompassed by the present invention.

45 Due to the degeneracy of the genetic code, two DNA sequences may differ and yet
encode identical amino acid sequences. The present invention thus provides isolated nucleic
acid molecules having a polynucleotide sequence encoding any of the Hu-Asp polypeptides of
50 the invention, wherein said polynucleotide sequence encodes a Hu-Asp polypeptide having
the complete amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or
fragments thereof.

Also provided herein are purified Hu-Asp polypeptides, both recombinant and non-recombinant. Most importantly, methods to produce Hu-Asp2 polypeptides in active form are provided. These include production of Hu-Asp2 polypeptides and variants thereof in bacterial cells, insect cells, and mammalian cells, also in forms that allow secretion of the Hu-Asp2 polypeptide from bacterial, insect or mammalian cells into the culture medium, also methods to produce variants of Hu-Asp2 polypeptide incorporating amino acid tags that facilitate subsequent purification. In a preferred embodiment of the invention the Hu-Asp2 polypeptide is converted to a proteolytically active form either in transformed cells or after purification and cleavage by a second protease in a cell-free system, such active forms of the Hu-Asp2 polypeptide beginning with the N-terminal sequence TQHGIR or ETDEEP. Variants and derivatives, including fragments, of Hu-Asp proteins having the native amino acid sequences given in SEQ ID Nos: 2, 4, and 6 that retain any of the biological activities of Hu-Asp are also within the scope of the present invention. Of course, one of ordinary skill in the art will readily be able to determine whether a variant, derivative, or fragment of a Hu-Asp protein displays Hu-Asp activity by subjecting the variant, derivative, or fragment to a standard aspartyl protease assay. Fragments of Hu-Asp within the scope of this invention include those that contain the active site domain containing the amino acid sequence DTG, fragments that contain the active site domain amino acid sequence DSG, fragments containing both the DTG and DSG active site sequences, fragments in which the spacing of the DTG and DSG active site sequences has been lengthened, fragments in which the spacing has been shortened. Also within the scope of the invention are fragments of Hu-Asp in which the transmembrane domain has been removed to allow production of Hu-Asp2 in a soluble form. In another embodiment of the invention, the two halves of Hu-Asp2, each containing a single active site DTG or DSG sequence can be produced independently as recombinant polypeptides, then combined in solution where they reconstitute an active protease.

Hu-Asp variants may be obtained by mutation of native Hu-Asp-encoding nucleotide sequences, for example. A Hu-Asp variant, as referred to herein, is a polypeptide substantially homologous to a native Hu-Asp polypeptide but which has an amino acid sequence different from that of native Hu-Asp because of one or more deletions, insertions, or substitutions in the amino acid sequence. The variant amino acid or nucleotide sequence is preferably at least about 80% identical, more preferably at least about 90% identical, and most preferably at least about 95% identical, to a native Hu-Asp sequence. Thus, a variant nucleotide sequence which contains, for example, 5 point mutations for every one hundred

5 nucleotides, as compared to a native Hu-Asp gene, will be 95% identical to the native protein.
The percentage of sequence identity, also termed homology, between a native and a variant
Hu-Asp sequence may also be determined, for example, by comparing the two sequences
using any of the computer programs commonly employed for this purpose, such as the Gap
10 5 program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer
Group, University Research Park, Madison Wisconsin), which uses the algorithm of Smith
and Waterman (*Adv. Appl. Math.* 2: 482-489 (1981)).

15 Alterations of the native amino acid sequence may be accomplished by any of a
number of known techniques. For example, mutations may be introduced at particular
10 locations by procedures well known to the skilled artisan, such as oligonucleotide-directed
mutagenesis, which is described by Walder *et al.* (*Gene* 42:133 (1986)); Bauer *et al.* (*Gene*
37:73 (1985)); Craik (*BioTechniques*, January 1985, pp. 12-19); Smith *et al.* (*Genetic*
20 *Engineering: Principles and Methods*, Plenum Press (1981)); and U.S. Patent Nos. 4,518,584
and 4,737,462.

25 Hu-Asp variants within the scope of the invention may comprise conservatively
substituted sequences, meaning that one or more amino acid residues of a Hu-Asp polypeptide
are replaced by different residues that do not alter the secondary and/or tertiary structure of the
Hu-Asp polypeptide. Such substitutions may include the replacement of an amino acid by a
30 residue having similar physicochemical properties, such as substituting one aliphatic residue
(Ile, Val, Leu or Ala) for another, or substitution between basic residues Lys and Arg, acidic
20 residues Glu and Asp, amide residues Gln and Asn, hydroxyl residues Ser and Tyr, or
aromatic residues Phe and Tyr. Further information regarding making phenotypically silent
amino acid exchanges may be found in Bowie *et al.*, *Science* 247:1306-1310 (1990). Other
Hu-Asp variants which might retain substantially the biological activities of Hu-Asp are those
25 where amino acid substitutions have been made in areas outside functional regions of the
protein.

40 In another aspect, the invention provides an isolated nucleic acid molecule comprising
a polynucleotide which hybridizes under stringent conditions to a portion of the nucleic acid
molecules described above, *e.g.*, to at least about 15 nucleotides, preferably to at least about
45 30 nucleotides, more preferably to at least about 30 nucleotides, and still more preferably to at
least about from 30 to at least about 100 nucleotides, of one of the previously described
nucleic acid molecules. Such portions of nucleic acid molecules having the described lengths
50 refer to, *e.g.*, at least about 15 contiguous nucleotides of the reference nucleic acid molecule.

5 By stringent hybridization conditions is intended overnight incubation at about 42°C for about 2.5 hours in 6 X SSC/0.1% SDS, followed by washing of the filters in 1.0 X SSC at 65°C, 0.1% SDS.

10 Fragments of the Hu-Asp-encoding nucleic acid molecules described herein, as well as 5 polynucleotides capable of hybridizing to such nucleic acid molecules may be used as a probe or as primers in a polymerase chain reaction (PCR). Such probes may be used, *e.g.*, to detect the presence of Hu-Asp nucleic acids in *in vitro* assays, as well as in Southern and northern blots. Cell types expressing Hu-Asp may also be identified by the use of such probes. Such 15 procedures are well known, and the skilled artisan will be able to choose a probe of a length suitable to the particular application. For PCR, 5' and 3' primers corresponding to the termini of a desired Hu-Asp nucleic acid molecule are employed to isolate and amplify that sequence using conventional techniques.

20 Other useful fragments of the Hu-Asp nucleic acid molecules are antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence capable of binding to a 25 target Hu-Asp mRNA (using a sense strand), or Hu-Asp DNA (using an antisense strand) sequence. In a preferred embodiment of the invention these Hu-Asp antisense oligonucleotides reduce Hu-Asp mRNA and consequent production of Hu-Asp polypeptides.

30 In another aspect, the invention includes Hu-Asp polypeptides with or without associated native pattern glycosylation. Both Hu-Asp1 and Hu-Asp2 have canonical acceptor 20 sites for Asn-linked sugars, with Hu-Asp1 having two of such sites, and Hu-Asp2 having four. Hu-Asp expressed in yeast or mammalian expression systems (discussed below) may be similar to or significantly different from a native Hu-Asp polypeptide in molecular weight and glycosylation pattern. Expression of Hu-Asp in bacterial expression systems will provide 35 non-glycosylated Hu-Asp.

40 The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Hu-Asp polypeptides may be recovered and purified from tissues, cultured cells, or recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, 45 phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, and high 30 performance liquid chromatography (HPLC). In a preferred embodiment, an amino acid tag is added to the Hu-Asp polypeptide using genetic engineering techniques that are well known to 50 practitioners of the art which include addition of six histidine amino acid residues to allow

5 purification by binding to nickel immobilized on a suitable support, epitopes for polyclonal or
monoclonal antibodies including but not limited to the T7 epitope, the myc epitope, and the
V5a epitope, and fusion of Hu-Asp2 to suitable protein partners including but not limited to
10 glutathione-S-transferase or maltose binding protein. In a preferred embodiment these
5 additional amino acid sequences are added to the C-terminus of Hu-Asp but may be added to
the N-terminus or at intervening positions within the Hu-Asp2 polypeptide.

The present invention also relates to vectors comprising the polynucleotide molecules
15 of the invention, as well as host cell transformed with such vectors. Any of the polynucleotide
molecules of the invention may be joined to a vector, which generally includes a selectable
10 marker and an origin of replication, for propagation in a host. Because the invention also
provides Hu-Asp polypeptides expressed from the polynucleotide molecules described above,
20 vectors for the expression of Hu-Asp are preferred. The vectors include DNA encoding any of
the Hu-Asp polypeptides described above or below, operably linked to suitable transcriptional
or translational regulatory sequences, such as those derived from a mammalian, microbial,
25 viral, or insect gene. Examples of regulatory sequences include transcriptional promoters,
operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which
control transcription and translation. Nucleotide sequences are operably linked when the
regulatory sequence functionally relates to the DNA encoding Hu-Asp. Thus, a promoter
30 nucleotide sequence is operably linked to a Hu-Asp DNA sequence if the promoter nucleotide
20 sequence directs the transcription of the Hu-Asp sequence.

Selection of suitable vectors to be used for the cloning of polynucleotide molecules
35 encoding Hu-Asp, or for the expression of Hu-Asp polypeptides, will of course depend upon
the host cell in which the vector will be transformed, and, where applicable, the host cell from
which the Hu-Asp polypeptide is to be expressed. Suitable host cells for expression of Hu-
25 Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is
discussed below.

The Hu-Asp polypeptides to be expressed in such host cells may also be fusion
40 proteins which include regions from heterologous proteins. Such regions may be included to
allow, e.g., secretion, improved stability, or facilitated purification of the polypeptide. For
45 example, a sequence encoding an appropriate signal peptide can be incorporated into
30 expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused
in-frame to the Hu-Asp sequence so that Hu-Asp is translated as a fusion protein comprising
50 the signal peptide. A signal peptide that is functional in the intended host cell promotes

5 extracellular secretion of the Hu-Asp polypeptide. Preferably, the signal sequence will be cleaved from the Hu-Asp polypeptide upon secretion of Hu-Asp from the cell. Non-limiting examples of signal sequences that can be used in practicing the invention include the yeast I-factor and the honeybee melatin leader in sf9 insect cells.

10 5 In a preferred embodiment, the Hu-Asp polypeptide will be a fusion protein which includes a heterologous region used to facilitate purification of the polypeptide. Many of the available peptides used for such a function allow selective binding of the fusion protein to a binding partner. For example, the Hu-Asp polypeptide may be modified to comprise a peptide to form a fusion protein which specifically binds to a binding partner, or peptide tag.
15 Non-limiting examples of such peptide tags include the 6-His tag, thioredoxin tag, hemagglutinin tag, GST tag, and OmpA signal sequence tag. As will be understood by one of skill in the art, the binding partner which recognizes and binds to the peptide may be any molecule or compound including metal ions (e.g., metal affinity columns), antibodies, or fragments thereof, and any protein or peptide which binds the peptide, such as the FLAG tag.

20 15 Suitable host cells for expression of Hu-Asp polypeptides includes prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of Hu-Asp include bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. For expression in, e.g., *E. coli*, a Hu-Asp polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host. The N-terminal Met may optionally then be cleaved from the expressed Hu-Asp polypeptide. Other N-terminal amino acid residues can be added to the Hu-Asp polypeptide to facilitate expression in *Escherichia coli* including but not limited to the T7 leader sequence, the T7-caspase 8 leader sequence, as well as others leaders including tags for purification such as the 6-His tag (Example 9).
25 20 Asp polypeptides expressed in *E. coli* may be shortened by removal of the cytoplasmic tail, the transmembrane domain, or the membrane proximal region. Hu-Asp polypeptides expressed in *E. coli* may be obtained in either a soluble form or as an insoluble form which may or may not be present as an inclusion body. The insoluble polypeptide may be rendered soluble by guanidine HCl, urea or other protein denaturants, then refolded into a soluble form before or after purification by dilution or dialysis into a suitable aqueous buffer. If the inactive proform of the Hu-Asp was produced using recombinant methods, it may be rendered active by cleaving off the prosegment with a second suitable protease such as human immunodeficiency virus protease.
30 35 40 45 50

5 Expression vectors for use in prokaryotic hosts generally comprises one or more phenotypic selectable marker genes. Such genes generally encode, *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, 10 pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), pET vectors (Novagen) and pQE vectors (Qiagen).

Hu-Asp may also be expressed in yeast host cells from genera including *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Yeast vectors will often contain an origin of replication sequence from a 2T yeast 15 plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of Hu-Asp polypeptides 20 expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the Hu-Asp-encoding nucleotide sequence.

Insect host cell culture systems may also be used for the expression of Hu-Asp polypeptides. In a preferred embodiment, the Hu-Asp polypeptides of the invention are 30 expressed using an insect cell expression system (*see* Example 10). Additionally, a baculovirus expression system can be used for expression in insect cells as reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

In another preferred embodiment, the Hu-Asp polypeptide is expressed in mammalian host cells. Non-limiting examples of suitable mammalian cell lines include the COS-7 line of 35 monkey kidney cells (Gluzman *et al.*, *Cell* 23:175 (1981)), human embryonic kidney cell line 293, and Chinese hamster ovary (CHO) cells. Preferably, Chinese hamster ovary (CHO) cells are used for expression of Hu-Asp proteins (Example 11).

The choice of a suitable expression vector for expression of the Hu-Asp polypeptides of the invention will of course depend upon the specific mammalian host cell to be used, and 45 is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A preferred vector for expression of Hu-Asp polypeptides is pcDNA3.1-Hygro (Invitrogen). Expression vectors for use in 50 mammalian host cells may include transcriptional and translational control sequences derived

5 from viral genomes. Commonly used promoter sequences and enhancer sequences which
may be used in the present invention include, but are not limited to, those derived from human
cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40).
Methods for the construction of mammalian expression vectors are disclosed, for example, in
10 Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983)); Cosman *et al.* (*Mol. Immunol.* 23:935
(1986)); Cosman *et al.* (*Nature* 312:768 (1984)); EP-A-0367566; and WO 91/18982.

The polypeptides of the present invention may also be used to raise polyclonal and
monoclonal antibodies, which are useful in diagnostic assays for detecting Hu-Asp
15 polypeptide expression. Such antibodies may be prepared by conventional techniques. See,
for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, N.Y., (1988); *Monoclonal Antibodies, Hybridomas: A
New Dimension in Biological Analyses*, Kennet *et al.* (eds.), Plenum Press, New York (1980).
20 Synthetic peptides comprising portions of Hu-Asp containing 5 to 20 amino acids may also be
used for the production of polyclonal or monoclonal antibodies after linkage to a suitable
carrier protein including but not limited to keyhole limpet hemacyanin (KLH), chicken
ovalbumin, or bovine serum albumin using various cross-linking reagents including
carbodimides, glutaraldehyde, or if the peptide contains a cysteine, N-methylmaleimide. A
25 preferred peptide for immunization when conjugated to KLH contains the C-terminus of
Hu_Asp1 or Hu-Asp2 comprising QRRPRDPEVVNDESSLVRHRWK or
30 LRQQHDDFADDISLLK, respectively.
20 LRQQHDDFADDISLLK, respectively.

The Hu-Asp nucleic acid molecules of the present invention are also valuable for
chromosome identification, as they can hybridize with a specific location on a human
35 chromosome. Hu-Asp1 has been localized to chromosome 21, while Hu-Asp2 has been
localized to chromosome 11q23.3-24.1. There is a current need for identifying particular sites
25 on the chromosome, as few chromosome marking reagents based on actual sequence data
(repeat polymorphisms) are presently available for marking chromosomal location. Once a
40 sequence has been mapped to a precise chromosomal location, the physical position of the
sequence on the chromosome can be correlated with genetic map data. The relationship
between genes and diseases that have been mapped to the same chromosomal region can then
45 be identified through linkage analysis, wherein the coinheritance of physically adjacent genes
is determined. Whether a gene appearing to be related to a particular disease is in fact the
30 cause of the disease can then be determined by comparing the nucleic acid sequence between
50 affected and unaffected individuals.

5 In another embodiment, the invention relates to a method of assaying Hu-Asp function, specifically Hu-Asp2 function which involves incubating in solution the Hu-Asp polypeptide with a suitable substrate including but not limited to a synthetic peptide containing the β -
10 5 secretase cleavage site of APP, preferably one containing the mutation found in a Swedish kindred with inherited AD in which KM is changed to NL, such peptide comprising the sequence SEVNLDAEFR in an acidic buffering solution, preferably an acidic buffering solution of pH5.5 (see Example 12) using cleavage of the peptide monitored by high
15 performance liquid chromatography as a measure of Hu-Asp proteolytic activity. Preferred assays for proteolytic activity utilize internally quenched peptide assay substrates. Such suitable substrates include peptides which have attached a paired fluorphore and quencher including but not limited to coumarin and dinitrophenol, respectively, such that cleavage of the peptide by the Hu-Asp results in increased fluorescence due to physical separation of the fluorphore and quencher. Preferred colorimetric assays of Hu-Asp proteolytic activity utilize
20 other suitable substrates that include the P2 and P1 amino acids comprising the recognition site for cleavage linked to o-nitrophenol through an amide linkage, such that cleavage by the Hu-Asp results in an increase in optical density after altering the assay buffer to alkaline pH.

In another embodiment, the invention relates to a method for the identification of an agent that increases the activity of a Hu-Asp polypeptide selected from the group consisting of
30 Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

- (a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and
- (b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

40 whereby a higher level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has increased the activity of said Hu-Asp polypeptide. Such tests can be performed with Hu-Asp polypeptide in a cell free system and with cultured cells that express Hu-Asp as well as variants or isoforms thereof.

45 30 In another embodiment, the invention relates to a method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

5 (a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

10 (b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

whereby a lower level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has decreased the activity of said Hu-Asp polypeptide. Such tests can be performed with Hu-Asp polypeptide in a cell free system and with cultured cells that express Hu-Asp as well as variants or isoforms thereof.

15 In another embodiment, the invention relates to a novel cell line (HEK125.3 cells) for measuring processing of amyloid β peptide ($A\beta$) from the amyloid protein precursor (APP). The cells are stable transformants of human embryonic kidney 293 cells (HEK293) with a bicistronic vector derived from pIRES-EGFP (Clontech) containing a modified human APP cDNA, an internal ribosome entry site and an enhanced green fluorescent protein (EGFP) cDNA in the second cistron. The APP cDNA was modified by adding two lysine codons to the carboxyl terminus of the APP coding sequence. This increases processing of $A\beta$ peptide from human APP by 2-4 fold. This level of $A\beta$ peptide processing is 60 fold higher than is seen in nontransformed HEK293 cells. HEK125.3 cells will be useful for assays of compounds that inhibit $A\beta$ peptide processing. This invention also includes addition of two lysine residues to the C-terminus of other APP isoforms including the 751 and 770 amino acid isoforms, to isoforms of APP having mutations found in human AD including the Swedish KM \rightarrow NL and V717 \rightarrow F mutations, to C-terminal fragments of APP, such as those beginning with the β -secretase cleavage site, to C-terminal fragments of APP containing the β -secretase cleavage site which have been operably linked to an N-terminal signal peptide for membrane insertion and secretion, and to C-terminal fragments of APP which have been operably linked to an N-terminal signal peptide for membrane insertion and secretion and a reporter sequence including but not limited to green fluorescent protein or alkaline phosphatase, such that β -secretase cleavage releases the reporter protein from the surface of cells expressing the polypeptide.

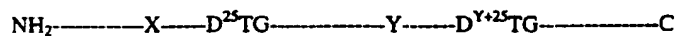
25 Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

EXAMPLES

Example 1: Development of a Search Algorithm Useful for the Identification of Aspartyl Proteases, and Identification of *C. elegans* Aspartyl Protease Genes in Wormpep 12:

Materials and Methods:

Classical aspartyl proteases such as pepsin and renin possess a two-domain structure which folds to bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. The DTG or DSG active site motif appears at about residue 25-30 in the enzyme, but at about 65-70 in the proenzyme (prorenin, pepsinogen). This motif appears again about 150-200 residues downstream. The proenzyme is activated by cleavage of the N-terminal prodomain. This pattern exemplifies the double domain structure of the modern day aspartyl enzymes which apparently arose by gene duplication and divergence. Thus;



where X denotes the beginning of the enzyme, following the N-terminal prodomain, and Y denotes the center of the molecule where the gene repeat begins again.

In the case of the retroviral enzymes such as the HIV protease, they represent only a half of the two-domain structures of well-known enzymes like pepsin, cathepsin D, renin, etc. They have no prosegment, but are carved out of a polyprotein precursor containing the *gag* and *pol* proteins of the virus. They can be represented by:



This "monomer" only has about 100 aa, so is extremely parsimonious as compared to the other aspartyl protease "dimers" which have of the order of 330 or so aa, not counting the N-terminal prodomain.

The limited length of the eukaryotic aspartyl protease active site motif makes it difficult to search EST collections for novel sequences. EST sequences typically average 250 nucleotides, and so in this case would be unlikely to span both aspartyl protease active site motifs. Instead, we turned to the *C. elegans* genome. The *C. elegans* genome is estimated to contain around 13,000 genes. Of these, roughly 12,000 have been sequenced and the corresponding hypothetical open reading frame (ORF) has been placed in the database Wormpep12. We used this database as the basis for a whole genome scan of a higher eukaryote for novel aspartyl proteases, using an algorithm that we developed

specifically for this purpose. The following AWK script for locating proteins containing two DTG or DSG motifs was used for the search, which was repeated four times to recover all pairwise combinations of the aspartyl motif.

```

BEGIN{RS=">"}          /* defines ">" as record separator for FASTA format */
{
  pos = index($0,"DTG")  /* finds "DTG" in record */
  if (pos>0) {
    rest = substr($0,pos+3) /*get rest of record after first DTG*/
    pos2 = index(rest,"DTG") /*find second DTG*/
    if (pos2>0) printf ("%s%s\n",">",$0) /*report hits*/
  }
}

```

The AWK script shown above was used to search Wormpep12, which was downloaded from ftp.sanger.ac.uk/pub/databases/wormpep, for sequence entries containing at least two DTG or DSG motifs. Using AWK limited each record to 3000 characters or less. Thus, 35 or so larger records were eliminated manually from Wormpep12 as in any case these were unlikely to encode aspartyl proteases.

Results and Discussion:

The Wormpep 12 database contains 12,178 entries, although some of these (<10%) represent alternatively spliced transcripts from the same gene. Estimates of the number of genes encoded in the *C. elegans* genome is on the order of 13,000 genes, so Wormpep12 may be estimated to cover greater than 90% of the *C. elegans* genome.

Eukaryotic aspartyl proteases contain a two-domain structure, probably arising from ancestral gene duplication. Each domain contains the active site motif D(S/T)G located from 20-25 amino acid residues into each domain. The retroviral (e.g., HIV protease) or retrotransposon proteases are homodimers of subunits which are homologous to a single eukaryotic aspartyl protease domain. An AWK script was used to search the Wormpep12 database for proteins in which the D(S/T)G motif occurred at least twice. This identified >60 proteins with two DTG or DSG motifs. Visual inspection was used to select proteins in which the position of the aspartyl domains was suggestive of a two-domain structure meeting the criteria described above.

In addition, the PROSITE eukaryotic and viral aspartyl protease active site pattern PS00141 was used to search Wormpep12 for candidate aspartyl proteases. (Bairoch A., Bucher P., Hofmann K., The PROSITE database: its status in 1997, *Nucleic Acids Res.* 24:217-221(1997)). This generated an overlapping set of Wormpep12 sequences. Of these,

5 seven sequences contained two DTG or DSG motifs and the PROSITE aspartyl protease active site pattern. Of these seven, three were found in the same cosmid clone (F21F8.3, F21F8.4, and F21F8.7) suggesting that they represent a family of proteins that arose by ancestral gene duplication. Two other ORFs with extensive homology to F21F8.3, F21F8.4
10 5 and F21F8.7 are present in the same gene cluster (F21F8.2 and F21F8.6), however, these contain only a single DTG motif. Exhaustive BLAST searches with these seven sequences against Wormpep12 failed to reveal additional candidate aspartyl proteases in the *C. elegans* genome containing two repeats of the DTG or DSG motif.

15 BLASTX search with each *C. elegans* sequence against SWISS-PROT, GenPep and
10 TREMBL revealed that R12H7.2 was the closest worm homologue to the known mammalian aspartyl proteases, and that T18H9.2 was somewhat more distantly related, while CEASP1, F21F8.3, F21F8.4, and F21F8.7 formed a subcluster which had the least
20 sequence homology to the mammalian sequences.

Discussion:

15 APP, the presenilins, and p35, the activator of cdk5, all undergo intracellular
25 proteolytic processing at sites which conform to the substrate specificity of the HIV protease. Dysregulation of a cellular aspartyl protease with the same substrate specificity, might therefore provide a unifying mechanism for causation of the plaque and tangle
30 pathologies in AD. Therefore, we sought to identify novel human aspartyl proteases. A
20 whole genome scan in *C. elegans* identified seven open reading frames that adhere to the aspartyl protease profile that we had identified. These seven aspartyl proteases probably comprise the complete complement of such proteases in a simple, multicellular eukaryote.
35 These include four closely related aspartyl proteases unique to *C. elegans* which probably arose by duplication of an ancestral gene. The other three candidate aspartyl proteases
25 (T18H9.2, R12H7.2 and C11D2.2) were found to have homology to mammalian gene
40 sequences.

Example 2: Identification of Novel Human Aspartyl Proteases Using Database Mining by Genome Bridging

Materials and Methods:

Computer-assisted analysis of EST databases, cDNA, and predicted polypeptide sequences:

Exhaustive homology searches of EST databases with the CEASP1, F21F8.3, F21F8.4, and F21F8.7 sequences failed to reveal any novel mammalian homologues. TBLASTN searches with R12H7.2 showed homology to cathepsin D, cathepsin E, pepsinogen A, pepsinogen C and renin, particularly around the DTG motif within the active site, but also failed to identify any additional novel mammalian aspartyl proteases. This indicates that the *C. elegans* genome probably contains only a single lysosomal aspartyl protease which in mammals is represented by a gene family that arose through duplication and consequent modification of an ancestral gene.

TBLASTN searches with T18H9.2, the remaining *C. elegans* sequence, identified several ESTs which assembled into a contig encoding a novel human aspartyl protease (Hu-ASP1). As is described above in Example 1, BLASTX search with the Hu-ASP1 contig against SWISS-PROT revealed that the active site motifs in the sequence aligned with the active sites of other aspartyl proteases. Exhaustive, repetitive rounds of BLASTN searches against LifeSeq, LifeSeqFL, and the public EST collections identified 102 EST from multiple cDNA libraries that assembled into a single contig. The 51 sequences in this contig found in public EST collections also have been assembled into a single contig (THC213329) by The Institute for Genome Research (TIGR). The TIGR annotation indicates that they failed to find any hits in the database for the contig. Note that the TIGR contig is the reverse complement of the LifeSeq contig that we assembled. BLASTN search of Hu-ASP1 against the rat and mouse EST sequences in ZooSeq revealed one homologous EST in each database (Incyte clone 700311523 and IMAGE clone 313341, GenBank accession number W10530, respectively).

TBLASTN searches with the assembled DNA sequence for Hu-ASP1 against both LifeSeqFL and the public EST databases identified a second, related human sequence (Hu-Asp2) represented by a single EST (2696295). Translation of this partial cDNA sequence reveals a single DTG motif which has homology to the active site motif of a bovine aspartyl protease, NM1.

BLAST searches, contig assemblies and multiple sequence alignments were performed using the bioinformatics tools provided with the LifeSeq, LifeSeqFL and LifeSeq Assembled databases from Incyte. Predicted protein motifs were identified using either the ProSite dictionary (Motifs in GCG 9) or the Pfam database.

5 Full-length cDNA cloning of Hu-Asp1

The open reading frame of *C. elegans* gene T18H9.2CE was used to query Incyte LifeSeq and LifeSeq-FL databases and a single electronic assembly referred to as 1863920CE1 was detected. The 5' most cDNA clone in this contig, 1863920, was obtained from Incyte and completely sequenced on both strands. Translation of the open reading frame contained within clone 1863920 revealed the presence of the duplicated aspartyl protease active site motif (DTG/DSG) but the 5' end was incomplete. The remainder of the Hu-Asp1 coding sequence was determined by 5' Marathon RACE analysis using a human placenta Marathon ready cDNA template (Clontech). A 3'-antisense oligonucleotide primer specific for the 5' end of clone 1863920 was paired with the 5'-sense primer specific for the Marathon ready cDNA synthetic adaptor in the PCR. Specific PCR products were directly sequenced by cycle sequencing and the resulting sequence assembled with the sequence of clone 1863920 to yield the complete coding sequence of Hu-Asp-1 (SEQ ID No. 1).

Several interesting features are present in the primary amino acid sequence of Hu-Asp1 (Figure 1, SEQ ID No. 2). The sequence contains a signal peptide (residues 1-20 in SEQ ID No. 2), a pro-segment, and a catalytic domain containing two copies of the aspartyl protease active site motif (DTG/DSG). The spacing between the first and second active site motifs is about 200 residues which should correspond to the expected size of a single, eukaryotic aspartyl protease domain. More interestingly, the sequence contains a predicted transmembrane domain (residues 469-492 in SEQ ID No.2) near its C-terminus which suggests that the protease is anchored in the membrane. This feature is not found in any other aspartyl protease.

Cloning of a full-length Hu-Asp-2 cDNAs:

As is described above in Example 1, genome wide scan of the *Caenorhabditis elegans* database WormPep12 for putative aspartyl proteases and subsequent mining of human EST databases revealed a human ortholog to the *C. elegans* gene T18H9.2 referred to as Hu-Asp1. The assembled contig for Hu-Asp1 was used to query for human paralogs using the BLAST search tool in human EST databases and a single significant match

(2696295CE1) with approximately 60% shared identity was found in the LifeSeq FL database. Similar queries of either gb105PubEST or the family of human databases available from TIGR did not identify similar EST clones. cDNA clone 2696295, identified by single pass sequence analysis from a human uterus cDNA library, was obtained from Incyte and completely sequence on both strands. This clone contained an incomplete 1266 bp open-reading frame that encoded a 422 amino acid polypeptide but lacked an initiator ATG on the 5' end. Inspection of the predicted sequence revealed the presence of the duplicated aspartyl protease active site motif DTG/DSG, separated by 194 amino acid residues. Subsequent queries of later releases of the LifeSeq EST database identified an additional ESTs, sequenced from a human astrocyte cDNA library (4386993), that appeared to contain additional 5' sequence relative to clone 2696295. Clone 4386993 was obtained from Incyte and completely sequenced on both strands. Comparative analysis of clone 4386993 and clone 2696295 confirmed that clone 4386993 extended the open-reading frame by 31 amino acid residues including two in-frame translation initiation codons. Despite the presence of the two in-frame ATGs, no in-frame stop codon was observed upstream of the ATG indicating that the 4386993 may not be full-length. Furthermore, alignment of the sequences of clones 2696295 and 4386993 revealed a 75 base pair insertion in clone 2696295 relative to clone 4386993 that results in the insertion of 25 additional amino acid residues in 2696295. The remainder of the Hu-Asp2 coding sequence was determined by 5' Marathon RACE analysis using a human hippocampus Marathon ready cDNA template (Clontech). A 3'-antisense oligonucleotide primer specific for the shared 5'-region of clones 2696295 and 4386993 was paired with the 5'-sense primer specific for the Marathon ready cDNA synthetic adaptor in the PCR. Specific PCR products were directly sequenced by cycle sequencing and the resulting sequence assembled with the sequence of clones 2696295 and 4386993 to yield the complete coding sequence of Hu-Asp2(a) (SEQ ID No. 3) and Hu-Asp2(b) (SEQ ID No. 5), respectively.

Several interesting features are present in the primary amino acid sequence of Hu-Asp2(a) (Figure 2 and SEQ ID No. 4) and Hu-Asp-2(b) (Figure 3, SEQ ID No. 6). Both sequences contain a signal peptide (residues 1-21 in SEQ ID No. 4 and SEQ ID No. 6), a pro-segment, and a catalytic domain containing two copies of the aspartyl protease active site motif (DTG/DSG). The spacing between the first and second active site motifs is variable due to the 25 amino acid residue deletion in Hu-Asp-2(b) and consists of 168-*versus*-194 amino acid residues, for Hu-Asp2(b) and Hu-Asp-2(a), respectively. More

5 interestingly, both sequences contains a predicted transmembrane domain (residues 455-477 in SEQ ID No.4 and 430-452 in SEQ ID No. 6) near their C-termini which indicates that the protease is anchored in the membrane. This feature is not found in any other aspartyl protease except Hu-Asp1.

10 **Example 3. Molecular cloning of mouse Asp2 cDNA and genomic DNA.**
Cloning and characterization of murine Asp2 cDNA—The murine ortholog of Hu_Asp2 was cloned using a combination of cDNA library screening, PCR, and genomic cloning. Approximately 500,000 independent clones from a mouse brain cDNA library were screened using a ³²P-labeled coding sequence probe prepared from Hu_Asp2. Replicate
15 positives were subjected to DNA sequence analysis and the longest cDNA contained the entire 3' untranslated region and 47 amino acids in the coding region. PCR amplification of the same mouse brain cDNA library with an antisense oligonucleotide primer specific for the 5'—most cDNA sequence determined above and a sense primer specific for the 5' region of human Asp2 sequence followed by DNA sequence analysis gave an additional 980 bp of
20 the coding sequence. The remainder of the 5' sequence of murine Asp-2 was derived from genomic sequence (see below).

Isolation and sequence analysis of the murine Asp-2 gene—A murine EST sequence encoding a portion of the murine Asp2 cDNA was identified in the GenBank EST database using the BLAST search tool and the Hu-Asp2 coding sequence as the query. Clone
35 g3160898 displayed 88% shared identity to the human sequence over 352 bp.

40 Oligonucleotide primer pairs specific for this region of murine Asp2 were then synthesized and used to amplify regions of the murine gene. Murine genomic DNA, derived from strain 129/SvJ, was amplified in the PCR (25 cycles) using various primer sets specific for murine Asp2 and the products analyzed by agarose gel electrophoresis. The primer set Zoo-1 and
45 Zoo-4 amplified a 750 bp fragment that contained approximately 600 bp of intron sequence
25 based on comparison to the known cDNA sequence. This primer set was then used to

5 screen a murine BAC library by PCR, a single genomic clone was isolated and this cloned
was confirmed contain the murine Asp2 gene by DNA sequence analysis. Shotgun DNA
sequencing of this Asp2 genomic clone and comparison to the cDNA sequences of both
10 Hu_Asp2 and the partial murine cDNA sequences defined the full-length sequence of
murine Asp2 (SEQ ID No. 7). The predicted amino acid sequence of murine Asp2 (SEQ ID
No. 8) showed 96.4% shared identity (GCG BestFit algorithm) with 18/501 amino acid
15 residue substitutions compared to the human sequence (Figure 4).

Example 4: Tissue Distribution of Expression of Hu-Asp2 Transcripts:

Materials and Methods:

20 The tissue distribution of expression of Hu-Asp-2 was determined using multiple
tissue Northern blots obtained from Clontech (Palo Alto, CA). Incyte clone 2696295 in
the vector pINCY was digested to completion with *EcoRI*/*NotI* and the 1.8 kb cDNA insert
25 purified by preparative agarose gel electrophoresis. This fragment was radiolabeled to a
specific activity $> 1 \times 10^9$ dpm/ μ g by random priming in the presence of [α - 32 P-dATP]
(>3000 Ci/mmol, Amersham, Arlington Heights, IL) and Klenow fragment of DNA
polymerase I. Nylon filters containing denatured, size fractionated poly A⁺ RNAs isolated
30 from different human tissues were hybridized with 2×10^6 dpm/ml probe in ExpressHyb
buffer (Clontech, Palo Alto, CA) for 1 hour at 68 °C and washed as recommended by the
manufacture. Hybridization signals were visualized by autoradiography using BioMax XR
20 film (Kodak, Rochester, NY) with intensifying screens at -80 °C.

Results and Discussion:

Limited information on the tissue distribution of expression of Hu-Asp-2 transcripts
was obtained from database analysis due to the relatively small number of ESTs detected
40 using the methods described above (< 5). In an effort to gain further information on the
expression of the Hu-Asp2 gene, Northern analysis was employed to determine both the
25 size(s) and abundance of Hu-Asp2 transcripts. PolyA⁺ RNAs isolated from a series of
peripheral tissues and brain regions were displayed on a solid support following separation
under denaturing conditions and Hu-Asp2 transcripts were visualized by high stringency
45 hybridization to radiolabeled insert from clone 2696295. The 2696295 cDNA probe
visualized a constellation of transcripts that migrated with apparent sizes of 3.0kb, 4.4 kb
30 and 8.0 kb with the latter two transcript being the most abundant.

Across the tissues surveyed, Hu-Asp2 transcripts were most abundant in pancreas and brain with lower but detectable levels observed in all other tissues examined except thymus and PBLs. Given the relative abundance of Hu-Asp2 transcripts in brain, the regional expression in brain regions was also established. A similar constellation of transcript sizes were detected in all brain regions examined [cerebellum, cerebral cortex, occipital pole, frontal lobe, temporal lobe and putamen] with the highest abundance in the medulla and spinal cord.

Example 5: Northern Blot Detection of HuAsp-1 and HuAsp-2 Transcripts in Human Cell Lines:

A variety of human cell lines were tested for their ability to produce Hu-Asp1 and Asp2 mRNA. Human embryonic kidney (HEK-293) cells, African green monkey (Cos-7) cells, Chinese hamster ovary (CHO) cells, HELA cells, and the neuroblastoma cell line IMR-32 were all obtained from the ATCC. Cells were cultured in DME containing 10% FCS except CHO cells which were maintained in α -MEM/10% FCS at 37 °C in 5% CO₂ until they were near confluence. Washed monolayers of cells (3×10^7) were lysed on the dishes and poly A⁺ RNA extracted using the Qiagen Oligotex Direct mRNA kit. Samples containing 2 μ g of poly A⁺ RNA from each cell line were fractionated under denaturing conditions (glyoxal-treated), transferred to a solid nylon membrane support by capillary action, and transcripts visualized by hybridization with random-primed labeled (³²P) coding sequence probes derived from either Hu-Asp1 or Hu-Asp2. Radioactive signals were detected by exposure to X-ray film and by image analysis with a PhosphorImager.

The Hu-Asp1 cDNA probe visualized a similar constellation of transcripts (2.6 kb and 3.5 kb) that were previously detected in human tissues. The relative abundance determined by quantification of the radioactive signal was Cos-7 > HEK 292 = HELA > IMR32.

The Hu-Asp2 cDNA probe also visualized a similar constellation of transcripts compared to tissue (3.0 kb, 4.4 kb, and 8.0 kb) with the following relative abundance: HEK 293 > Cos 7 > IMR32 > HELA.

Example 6: Modification of APP to increase A β processing for in vitro screening

Human cell lines that process A β peptide from APP provide a means to screen in cellular assays for inhibitors of β - and γ -secretase. Production and release of A β peptide into the culture supernatant is monitored by an enzyme-linked immunosorbent assay (ELISA). Although expression of APP is widespread and both neural and non-neuronal cell lines

process and release A β peptide, levels of endogenous APP processing are low and difficult to detect by EIA. A β processing can be increased by expressing in transformed cell lines mutations of APP that enhance A β processing. We made the serendipitous observation that addition of two lysine residues to the carboxyl terminus of APP695 increases A β processing still further. This allowed us to create a transformed cell line that releases A β peptide into the culture medium at the remarkable level of 20,000 pg/ml.

Materials And Methods

Materials:

Human embryonic kidney cell line 293 (HEK293 cells) were obtained internally.

The vector pIRES-EGFP was purchased from Clontech. Oligonucleotides for mutation using the polymerase chain reaction (PCR) were purchased from Genosys. A plasmid containing human APP695 (SEQ ID No. 9 [nucleotide] and SEQ ID No. 10 [amino acid]) was obtained from Northwestern University Medical School. This was subcloned into pSK (Stratagene) at the *Not*I site creating the plasmid pAPP695.

Mutagenesis protocol:

The Swedish mutation (K670N, M671L) was introduced into pAPP695 using the Stratagene Quick Change Mutagenesis Kit to create the plasmid pAPP695NL (SEQ ID No. 11 [nucleotide] and SEQ ID No. 12 [amino acid]). To introduce a di-lysine motif at the C-terminus of APP695, the forward primer #276 5' GACTGACCACTCGACCAGTTTC (SEQ ID No. 47) was used with the "patch" primer #274 5' CGAATTAAATTCCAGCACACTGGCTACTTCTTGTCTGCATCTCAAAGAAC (SEQ ID No. 48) and the flanking primer #275 CGAATTAAATTCCAGCACACTGGCTA (SEQ ID No. 49) to modify the 3' end of the APP695 cDNA (SEQ ID No. 15 [nucleotide] and SEQ ID No. 16 [amino acid]). This also added a BstXI restriction site that will be compatible with the BstXI site in the multiple cloning site of pIRES-EGFP. PCR amplification was performed with a Clontech HF Advantage cDNA PCR kit using the polymerase mix and buffers supplied by the manufacturer. For "patch" PCR, the patch primer was used at 1/20th the molar concentration of the flanking primers. PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen). After digestion with restriction enzymes, products were separated on 0.8% agarose gels and then excised DNA fragments were purified using a QIAquick gel extraction kit (Qiagen).

To reassemble a modified APP695-Sw cDNA, the 5' *Not*I-BglII fragment of the APP695-Sw cDNA and the 3' BglII-BstXI APP695 cDNA fragment obtained by PCR were

ligated into pIRES-EGFP plasmid DNA opened at the NotI and BstXI sites. Ligations were performed for 5 minutes at room temperature using a Rapid DNA Ligation kit (Boehringer Mannheim) and transformed into Library Efficiency DH5a Competent Cells (GibcoBRL-Life Technologies). Bacterial colonies were screened for inserts by PCR amplification using primers #276 and #275. Plasmid DNA was purified for mammalian cell transfection using a QIAprep Spin Miniprep kit (Qiagen). The construct obtained was designated pMG125.3 (APPSW-KK, SEQ ID No. 17 [nucleotide] and SEQ ID No. 18 [amino acid]).

Mammalian Cell Transfection:

HEK293 cells for transfection were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cotransfections were performed using LipofectAmine (Gibco-BRL) with 3 µg pMG125.3 DNA and 9 µg pcDNA3.1 DNA per 10 x 10⁶ cells. Three days posttransfection, cells were passaged into medium containing G418 at a concentration of 400 µg/ml. After three days growth in selective medium, cells were sorted by their fluorescence.

Clonal Selection of 125.3 cells by FACS:

Cell samples were analyzed on an EPICS Elite ESP flow cytometer (Coulter, Hialeah, FL) equipped with a 488 nm excitation line supplied by an air-cooled argon laser. EGFP emission was measured through a 525 nm band-pass filter and fluorescence intensity was displayed on a 4-decade log scale after gating on viable cells as determined by forward and right angle light scatter. Single green cells were separated into each well of one 96 well plate containing growth medium without G418. After a four day recovery period, G418 was added to the medium to a final concentration of 400 µg/ml. After selection, 32% of the wells contained expanding clones. Wells with clones were expanded from the 96 well plate to a 24 well plate and then a 6 well plate with the fastest growing colonies chosen for expansion at each passage. The final cell line selected was the fastest growing of the final six passaged. This clone, designated 125.3, has been maintained in G418 at 400 µg/ml with passage every four days into fresh medium. No loss of Aβ production or EGFP fluorescence has been seen over 23 passages.

Aβ EIA Analysis (Double Antibody Sandwich ELISA for hAβ 1-40/42):

Cell culture supernatants harvested 48 hr after transfection were analyzed in a standard Aβ EIA as follows. Human Aβ 1-40 or 1-42 was measured using monoclonal antibody (mAb) 6E10 (Senetek, St. Louis, MO) and biotinylated rabbit antiserum 162 or

164 (New York State Institute for Basic Research, Staten Island, NY) in a double antibody sandwich ELISA. The capture antibody 6E10 is specific to an epitope present on the N-terminal amino acid residues 1-16 of hA β . The conjugated detecting antibodies 162 and 164 are specific for hA β 1-40 and 1-42, respectively. Briefly, a Nunc Maxisorp 96 well immunoplate was coated with 100 μ l/well of mAb 6E10 (5 μ g/ml) diluted in 0.1M carbonate-bicarbonate buffer, pH 9.6 and incubated at 4°C overnight. After washing the plate 3x with 0.01M DPBS (Modified Dulbecco's Phosphate Buffered Saline (0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01 M potassium chloride, pH 7.4) from Pierce, Rockford, IL) containing 0.05% of Tween-20 (DPBST), the plate was blocked for 60 min with 200 μ l of 10% normal sheep serum (Sigma) in 0.01M DPBS to avoid non-specific binding. Human A β 1-40 or 1-42 standards 100 μ l/well (Bachem, Torrance, CA) diluted, from a 1mg/ml stock solution in DMSO, in culture medium was added after washing the plate, as well as 100 μ l/well of sample, e.g. conditioned medium of transfected cells. The plate was incubated for 2 hours at room temperature and 4°C overnight. The next day, after washing the plate, 100 μ l/well biotinylated rabbit antiserum 162 1:400 or 164 1:50 diluted in DPBST + 0.5% BSA was added and incubated at room temperature for 1hr 15 min. Following washes, 100 μ l/well neutravidin-horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in DPBST was applied and incubated for 1 hr at room temperature. After the last washes 100 μ l/well of o-phenylenediamine dihydrochloride (Sigma Chemicals, St. Louis, MO) in 50mM citric acid/100mM sodium phosphate buffer (Sigma Chemicals, St. Louis, MO), pH 5.0, was added as substrate and the color development was monitored at 450nm in a kinetic microplate reader for 20 min. using Soft max Pro software. All standards and samples were run in triplicates. The samples with absorbance values falling within the standard curve were extrapolated from the standard curves using Soft max Pro software and expressed in pg/ml culture medium.

Results:

Addition of two lysine residues to the carboxyl terminus of APP695 greatly increases A β processing in HEK293 cells as shown by transient expression (Table 1). Addition of the di-lysine motif to APP695 increases A β processing to that seen with the APP695 containing the Swedish mutation. Combining the di-lysine motif with the Swedish mutation further increases processing by an additional 2.8 fold.

Cotransformation of HEK293 cells with pMG125.3 and pcDNA3.1 allowed dual selection of transformed cells for G418 resistance and high level expression of EGFP. After clonal selection by FACS, the cell line obtained, produces a remarkable 20,000 pg A β peptide per ml of culture medium after growth for 36 hr in 24 well plates. Production of A β peptide under various growth conditions is summarized in Table 2.

TABLE 1. Release of A β peptide into the culture medium 48 hr after transient transfection of HEK293 cells with the indicated vectors containing wildtype or modified APP. Values tabulated are mean + SD and P-value for pairwise comparison using Student's t-test assuming unequal variances.

APP Construct	A β 1-40 peptide (pg/ml)	Fold Increase	P-value
pIRES-EGFP vector	147 + 28	1.0	
wt APP695 (142.3)	194 + 15	1.3	0.051
wt APP695-KK (124.1)	424 + 34	2.8	3 x 10 ⁻⁵
APP695-Sw (143.3)	457 + 65	3.1	2 x 10 ⁻³
APP695-SwKK (125.3)	1308 + 98	8.9	3 x 10 ⁻⁴

TABLE 2. Release of A β peptide from HEK125.3 cells under various growth conditions.

Type of Culture Plate	Volume of Medium	Duration of Culture	Ab 1-40 (pg/ml)	Ab 1-42 (pg/ml)
24 well plate	400 μ l	36 hr	28,036	1,439

Example 7: Antisense oligomer inhibition of Abeta processing in HEK125.3 cells

The sequences of Hu-Asp1 and Hu-Asp2 were provided to Sequitur, Inc (Natick, MA) for selection of targeted sequences and design of 2nd generation chimeric antisense oligomers using proprietary technology (Sequitur Ver. D Pat pending #3002). Antisense oligomers Lot# S644, S645, S646 and S647 were targeted against Asp1. Antisense oligomers Lot# S648, S649, S650 and S651 were targeted against Asp2. Control antisense oligomers Lot# S652, S653, S655, and S674 were targeted against an irrelevant gene and antisense oligomers Lot #S656, S657, S658, and S659 were targeted against a second irrelevant gene.

For transfection with the antisense oligomers, HEK125.3 cells were grown to about 50% confluence in 6 well plates in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum. A stock solution of oligofectin G (Sequitur Inc., Natick, MA) at 2 mg/ml was diluted to 50 μ g/ml in serum free MEM. Separately, the antisense oligomer stock solution at 100 μ M was diluted to 800 nM in Opti-MEM (GIBCO-BRL, Grand

Island, NY). The diluted stocks of oligofectin G and antisense oligomer were then mixed at a ratio of 1:1 and incubated at room temperature. After 15 min incubation, the reagent was diluted 10 fold into MEM containing 10% fetal calf serum and 2 ml was added to each well of the 6 well plate after first removing the old medium. After transfection, cells were grown in the continual presence of the oligofectin G/antisense oligomer. To monitor Ab peptide release, 400 μ l of conditioned medium was removed periodically from the culture well and replaced with fresh medium beginning 24 hr after transfection. Data reported are from culture supernatants harvested 48 hr after transfection.

Results:

The 16 different antisense oligomers obtained from Sequitur Inc were transfected separately into HEK125.3 cells to determine their affect on A β peptide processing. Only antisense oligomers targeted against Asp1 & Asp2 reduced Abeta processing by HEK125.3 cells with those targeted against Asp2 having a greater inhibitory effect. Both A β (1-40) and A β (1-42) were inhibited by the same degree. In Table 3, percent inhibition is calculated with respect to untransfected cells. Antisense oligomer reagents giving greater than 50% inhibition are marked with an asterisk. Of the reagents tested, 3 of 4 antisense oligomers targeted against ASP1 gave an average 52% inhibition of A β 1-40 processing and 47% inhibition of A β 1-42 processing. For ASP2, 4 of 4 antisense oligomers gave greater than 50% inhibition with an average inhibition of 62% for A β 1-40 processing and 60% for A β 1-42 processing.

Table 3. Inhibition of A β peptide release from HEK125.3 cells treated with antisense oligomers.

Gene Targeted	Antisense Oligomer	Abeta (1-40)	Abeta (1-42)
Asp1-1	S 644	62%*	56%*
Asp1-2	S 645	41%*	38%*
Asp1-3	S646	52%*	46%*
Asp1-4	S647	6%	25%
Asp2-1	S648	71%*	67%*
Asp2-2	S649	83%*	76%*
Asp2-3	S650	46%*	50%*
Asp2-4	S651	47%*	46%*
Con1-1	S652	13%	18%
Con1-2	S653	35%	30%
Con1-3	S655	9%	18%
Con1-4	S674	29%	18%
Con2-1	S656	12%	18%
Con2-2	S657	16%	19%
Con2-3	S658	8%	35%

WO 00/17369

PCT/US99/20881

Con2-4

S659

3%

18%

5

10

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Example 8. Demonstration of Hu-Asp2 β -Secretase Activity in Cultured Cells

Several mutations in APP associated with early onset Alzheimer's disease have been shown to alter A β peptide processing. These flank the N- and C-terminal cleavage sites that release A β from APP. These cleavage sites are referred to as the β -secretase and γ -secretase cleavage sites, respectively. Cleavage of APP at the β -secretase site creates a C-terminal fragment of APP containing 99 amino acids of 11,145 daltons molecular weight. The Swedish KM \rightarrow NL mutation immediately upstream of the β -secretase cleavage site causes a general increase in production of both the 1-40 and 1-42 amino acid forms of A β peptide. The London VF mutation (V717 \rightarrow F in the APP770 isoform) has little effect on total A β peptide production, but appears to preferentially increase the percentage of the longer 1-42 amino acid form of A β peptide by affecting the choice of γ -secretase cleavage site used during APP processing. Thus, we sought to determine if these mutations altered the amount and type of A β peptide produced by cultured cells cotransfected with a construct directing expression of Hu-Asp2.

Two experiments were performed which demonstrate Hu-Asp2 β -secretase activity in cultured cells. In the first experiment, treatment of HEK125.3 cells with antisense oligomers directed against Hu-Asp2 transcripts as described in Example 7 was found to decrease the amount of the C-terminal fragment of APP created by β -secretase cleavage (CTF99) (Figure 9). This shows that Hu-Asp2 acts directly or indirectly to facilitate β -secretase cleavage. In the second experiment, increased expression of Hu-Asp2 in transfected mouse Neuro2A cells is shown to increase accumulation of the CTF99 β -secretase cleavage fragment (Figure 10). This increase is seen most easily when a mutant APP-KK clone containing a C-terminal di-lysine motif is used for transfection. A further increase is seen when Hu-Asp2 is cotransfected with APP-Sw-KK containing the Swedish mutation KM \rightarrow NL. The Swedish mutation is known to increase cleavage of APP by the β -secretase.

5 A second set of experiments demonstrate Hu-Asp2 facilitates γ -secretase activity in
cotransfection experiments with human embryonic kidney HEK293 cells. Cotransfection of
Hu-Asp2 with an APP-KK clone greatly increases production and release of soluble A β 1-40
10 and A β 1-42 peptides from HEK293 cells. There is a proportionately greater increase in the
5 release of A β 1-42. A further increase in production of A β 1-42 is seen when Hu-Asp2 is
cotransfected with APP-VF (SEQ ID No. 13 [nucleotide] and SEQ ID No. 14 [amino acid])
or APP-VF-KK (SEQ ID No. 19 [nucleotide] and SEQ ID No. 20 [amino acid]) clones
15 containing the London mutation V717→F. The V717→F mutation is known to alter
cleavage specificity of the APP γ -secretase such that the preference for cleavage at the A β 42
10 site is increased. Thus, Asp2 acts directly or indirectly to facilitate γ -secretase processing of
APP at the β 42 cleavage site.

20 Materials

Antibodies 6E10 and 4G8 were purchased from Senetek (St. Louis, MO). Antibody 369
25 was obtained from the laboratory of Paul Greengard at the Rockefeller University.
15 Antibody C8 was obtained from the laboratory of Dennis Selkoe at the Harvard Medical
School and Brigham and Women's Hospital.

APP Constructs used

30 The APP constructs used for transfection experiments comprised the following

20	APP	wild-type APP695 (SEQ ID No. 9 and No. 10)
	APP-Sw	APP695 containing the Swedish KM→NL mutation (SEQ ID No. 11 and No. 12),
35	APP-VF	APP695 containing the London V→F mutation (SEQ ID No. 13 and No. 14)
25	APP-KK	APP695 containing a C-terminal KK motif (SEQ ID No. 15 and No. 16),
40	APP-Sw-KK	APP695-Sw containing a C-terminal KK motif (SEQ ID No. 17 and No. 18),
30	APP-VF-KK	APP695-VF containing a C-terminal KK motif (SEQ ID No. 19 and No. 20).

45 These were inserted into the vector pIRES-EGFP (Clontech, Palo Alto CA) between the
NotI and BstXI sites using appropriate linker sequences introduced by PCR.

50 *Transfection of antisense oligomers or plasmid DNA constructs in HEK293 cells,
HEK125.3 cells and Neuro-2A cells,*

Human embryonic kidney HEK293 cells and mouse Neuro-2a cells were transfected with expression constructs using the Lipofectamine Plus reagent from Gibco/BRL. Cells were seeded in 24 well tissue culture plates to a density of 70-80% confluence. Four wells per plate were transfected with 2 µg DNA (3:1, APP:cotransfectant), 8 µl Plus reagent, and 4 µl Lipofectamine in OptiMEM. OptiMEM was added to a total volume of 1 ml, distributed 200 µl per well and incubated 3 hours. Care was taken to hold constant the ratios of the two plasmids used for cotransfection as well as the total amount of DNA used in the transfection. The transfection media was replaced with DMEM, 10%FBS, NaPyruvate, with antibiotic/antimycotic and the cells were incubated under normal conditions (37°, 5% CO₂) for 48 hours. The conditioned media were removed to polypropylene tubes and stored at -80°C until assayed for the content of Aβ1-40 and Aβ1-42 by EIA as described in the preceding examples. Transfection of antisense oligomers into HEK125.3 cells was as described in Example 7.

Preparation of cell extracts, Western blot protocol

Cells were harvested after being transfected with plasmid DNA for about 60 hours. First, cells were transferred to 15-ml conical tube from the plate and centrifuged at 1,500 rpm for 5 min to remove the medium. The cell pellets were washed with PBS for one time. We then lysed the cells with lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium vanadate and 1% NP-40). The lysed cell mixtures were centrifuged at 5000 rpm and the supernatant was stored at -20°C as the cell extracts. Equal amounts of extracts from HEK125.3 cells transfected with the Asp2 antisense oligomers and controls were precipitated with antibody 369 that recognizes the C-terminus of APP and then CTF99 was detected in the immunoprecipitate with antibody 6E10. The experiment was repeated using C8, a second precipitating antibody that also recognizes the C-terminus of APP. For Western blot of extracts from mouse Neuro-2a cells cotransfected with Hu-Asp2 and APP-KK, APP-Sw-KK, APP-VF-KK or APP-VF, equal amounts of cell extracts were electrophoresed through 4-10% or 10-20% Tricine gradient gels (NOVEX, San Diego, CA). Full length APP and the CTF99 β-secretase product were detected with antibody 6E10.

Results

Transfection of HEK125.3 cells with Asp2-1 or Asp2-2 antisense oligomers reduces production of the CTF β -secretase product in comparison to cells similarly transfected with control oligomers having the reverse sequence (Asp2-1 reverse & Asp2-2 reverse)

In cotransfection experiments, cotransfection of Hu-Asp2 into mouse Neuro-2a cells with the APP-KK construct increased the formation of CTF99. This was further increased if Hu-Asp2 was coexpressed with APP-Sw-KK, a mutant form of APP containing the Swedish KM \rightarrow NL mutation that increases β -secretase processing.

Cotransfection of Hu-Asp2 with APP has little effect on A β 40 production but increases A β 42 production above background (Table 4). Addition of the di-lysine motif to the C-terminus of APP increases A β peptide processing about two fold, although A β 40 and A β 42 production remain quite low (352 pg/ml and 21 pg/ml, respectively). Cotransfection of Asp2 with APP-KK further increases both A β 40 and A β 42 production. The stimulation of A β 40 production by Hu-Asp2 is more than 3 fold, while production of A β 42 increases by more than 10 fold. Thus, cotransfection of Hu-Asp2 and APP-KK constructs preferentially increases A β 42 production.

The APP V717 \rightarrow F mutation has been shown to increase γ -secretase processing at the A β 42 cleavage site. Cotransfection of Hu-Asp2 with the APP-VF or APP-VF-KK constructs increased A β 42 production (a two fold increase with APP-VF and a four-fold increase with APP-VF-KK, Table 4), but had mixed effects on A β 40 production (a slight decrease with APP-VF, and a two fold increase with APP-VF-KK in comparison to the pcDNA cotransfection control. Thus, the effect of Asp2 on A β 42 production was proportionately greater leading to an increase in the ratio of A β 42/total A β . Indeed, the ratio of A β 42/total A β reaches a very high value of 42% in HEK293 cells cotransfected with Hu-Asp2 and APP-VF-KK.

Western blot showing reduction of CTF99 production by HEK125.3 cells transfected with antisense oligomers targeting the Hu-Asp2 mRNA. (right) Western blot showing increase in CTF99 production in mouse Neuro-2a cells cotransfected with Hu-Asp2 and APP-KK. A further increase in CTF99 production is seen in cells cotransfected with Hu-Asp2 and APP-Sw-KK.

Table 4. Results of cotransfecting Hu-Asp2 or pcDNA plasmid DNA with various APP constructs containing the V717→F mutation that modifies γ -secretase processing. Cotransfection with Asp2 consistently increases the ratio of A β 42/total A β . Values tabulated are A β peptide pg/ml.

	pcDNA Cotransfection			Asp2 Cotransfection		
	A β 40	A β 42	A β 42/Total	A β 40	A β 42	A β 42/Total
APP	192 \pm 18	<4	<2%	188 \pm 40	8 \pm 10	3.9%
APP-VF	118 \pm 15	15 \pm 19	11.5%	85 \pm 7	24 \pm 12	22.4%
APP-KK	352 \pm 24	21 \pm 6	5.5%	1062 \pm 101	226 \pm 49	17.5%
APP-VF-KK	230 \pm 31	88 \pm 24	27.7%	491 \pm 35	355 \pm 36	42%

Example 9. Bacterial expression of human Asp2L

Expression of recombinant Hu_Asp2L in E. coli.

Hu-Asp2L can be expressed in E. coli after addition of N-terminal sequences such as a T7 tag (SEQ ID No. 21 and No. 22) or a T7 tag followed by a caspase 8 leader sequence (SEQ ID No. 23 and No. 24). Alternatively, reduction of the GC content of the 5' sequence by site directed mutagenesis can be used to increase the yield of Hu-Asp2 (SEQ ID No. 25 and No. 26). In addition, Asp2 can be engineered with a proteolytic cleavage site (SEQ ID No. 27 and No. 28). To produce a soluble protein after expression and refolding, deletion of the transmembrane domain and cytoplasmic tail, or deletion of the membrane proximal region, transmembrane domain, and cytoplasmic tail is preferred.

Methods

PCR with primers containing appropriate linker sequences was used to assemble fusions of Asp2 coding sequence with N-terminal sequence modifications including a T7 tag (SEQ ID Nos. 21 and 22) or a T7-caspase 8 leader (SEQ ID Nos. 23 and 24). These constructs were cloned into the expression vector pet23a(+) [Novagen] in which a T7 promoter directs expression of a T7 tag preceding a sequence of multiple cloning sites. To clone Hu-Asp2 sequences behind the T7 leader of pet23a+, the following oligonucleotides were used for amplification of the selected Hu-Asp2 sequence:

#553=GTGGATCCACCCAGCACGGCATCCGGCTG (SEQ ID No. 35),

#554=GAAAGCTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ ID No. 36) which placed BamHI and HindIII sites flanking the 5' and 3' ends of the insert, respectively. The Asp2 sequence was amplified from the full length Asp2(b) cDNA cloned into pcDNA3.1 using the Advantage-GC cDNA PCR [Clontech] following the manufacturer's supplied protocol using annealing & extension at 68°C in a two-step PCR cycle for 25 cycles. The insert and vector were cut with BamHI and HindIII, purified by electrophoresis through an agarose gel, then ligated using the Rapid DNA Ligation kit [Boehringer Mannheim]. The ligation reaction was used to transform the E. coli strain JM109 (Promega) and colonies were picked for the purification of plasmid (Qiagen, Qiaprep minispin) and DNA sequence analysis. For inducible expression using induction with isopropyl b-D-thiogalactopyranoside (IPTG), the expression vector was transferred into E. coli strain BL21 (Statagene). Bacterial cultures were grown in LB broth in the presence of ampicillin at 100 ug/ml, and induced in log phase growth at an OD600 of 0.6-1.0 with 1 mM IPTG for 4 hour at 37°C. The cell pellet was harvested by centrifugation.

To clone Hu-Asp2 sequences behind the T7 tag and caspase leader (SEQ ID Nos. 23 and 24), the construct created above containing the T7-Hu-Asp2 sequence (SEQ ID Nos. 21 and 22) was opened at the BamHI site, and then the phosphorylated caspase 8 leader oligonucleotides #559=GATCGATGACTATCTCTGACTCTCCGCGTGAACAGGACG (SEQ ID No. 37), #560=GATCCGTCCTGTTACGCGGAGAGTCAGAGATAGTCATC (SEQ ID No. 38) were annealed and ligated to the vector DNA. The 5' overhang for each set of oligonucleotides was designed such that it allowed ligation into the BamHI site but not subsequent digestion with BamHI. The ligation reaction was transformed into JM109 as above for analysis of protein expression after transfer to E. coli strain BL21.

In order to reduce the GC content of the 5' terminus of asp2, a pair of antiparallel oligos were designed to change degenerate codon bases in 15 amino acid positions from G/C to A/T (SEQ ID Nos. 25 and 26). The new nucleotide sequence at the 5' end of asp2 did not change the encoded amino acid and was chosen to optimize E. Coli expression. The

sequence of the sense linker is 5'

CGGCATCCGGCTGCCCTGCGTAGCGGTCTGGGTGGTGCTCCACTGGGTCTGCG
TCTGCCCCGGGAGACCGACGAA G 3' (SEQ ID No. 39). The sequence of the antisense

linker is : 5'

CTTCGTCGGTCTCCCGGGGAGACGACGACCCAGTGGAGCACCACCCAGACCG
CTACGCAGGGGAGCCGGATGCCG 3' (SEQ ID No. 40). After annealing the
phosphorylated linkers together in 0.1 M NaCl-10 mM Tris, pH 7.4 they were ligated into
unique Cla I and Sma I sites in Hu-Asp2 in the vector pTAC. For inducible expression
using induction with isopropyl b-D-thiogalactopyranoside (IPTG), bacterial cultures were
grown in LB broth in the presence of ampicillin at 100 ug/ml, and induced in log phase
growth at an OD600 of 0.6-1.0 with 1 mM IPTG for 4 hour at 37°C. The cell pellet was
harvested by centrifugation.

To create a vector in which the leader sequences can be removed by limited
proteolysis with caspase 8 such that this liberates a Hu-Asp2 polypeptide beginning with
the N-terminal sequence GSFV (SEQ ID Nos. 27 and 28), the following procedure was
followed. Two phosphorylated oligonucleotides containing the caspase 8 cleavage site
IETD, #571=5'

GATCGATGACTATCTCTGACTCTCCGCTGGACTCTGGTATCGAAACCGACG
(SEQ ID No. 41) and #572=

GATCCGTCGGTTTCGATACCAGAGTCCAGCGGAGAGTCAGAGATAGTCATC
(SEQ ID No. 42) were annealed and ligated into pET23a+ that had been opened with
BamHI. After transformation into JM109, the purified vector DNA was recovered and
orientation of the insert was confirmed by DNA sequence analysis. +, the following
oligonucleotides were used for amplification of the selected Hu-Asp2 sequence:

#573=5'AAGGATCCTTTGTGGAGATGGTGGACAACCTG, (SEQ ID No. 43)

#554=GAAAGCTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ ID No. 44) which
placed BamHI and HindIII sites flanking the 5' and 3' ends of the insert, respectively. The
Asp2 sequence was amplified from the full length Asp2 cDNA cloned into pcDNA3.1
using the Advantage-GC cDNA PCR [Clontech] following the manufacturer's supplied

5 protocol using annealing & extension at 68°C in a two-step PCR cycle for 25 cycles. The
insert and vector were cut with BamHI and HindIII, purified by electrophoresis through an
10 agarose gel, then ligated using the Rapid DNA Ligation kit [Boehringer Mannheim]. The
ligation reaction was used to transform the E. coli strain JM109 [Promega] and colonies
5 were picked for the purification of plasmid (Qiagen, Qiaprep minispin) and DNA sequence
analysis. For inducible expression using induction with isopropyl b-D-
thiogalactopyranoside (IPTG), the expression vector was transferred into E. coli strain
15 BL21 (Statagene). Bacterial cultures were grown in LB broth in the presence of ampicillin
at 100 ug/ml, and induced in log phase growth at an OD600 of 0.6-1.0 with 1 mM IPTG for
10 4 hour at 37°C. The cell pellet was harvested by centrifugation.

To assist purification, a 6-His tag can be introduced into any of the above constructs
20 following the T7 leader by opening the construct at the BamHI site and then ligating in the
annealed, phosphorylated oligonucleotides containing the six histidine sequence
#565=GATCGCATCATCACCATCACCATG (SEQ ID No. 45),
15 #566=GATCCATGGTGATGGTGATGATGC (SEQ ID No. 46). The 5' overhang for each
set of oligonucleotides was designed such that it allowed ligation into the BamHI site but
25 not subsequent digestion with BamHI.

Preparation of Bacterial Pellet:

30 36.34g of bacterial pellet representing 10.8L of growth was dispersed into a total
volume of 200ml using a 20mm tissue homogenizer probe at 3000 to 5000 rpm in 2M KCl,
20 0.1M Tris, 0.05M EDTA, 1mM DTT. The conductivity adjusted to about 193mMhos with
water.
35 After the pellet was dispersed, an additional amount of the KCl solution was added,
bringing the total volume to 500 ml. This suspension was homogenized further for about 3
25 minutes at 5000 rpm using the same probe. The mixture was then passed through a Rannie
40 high-pressure homogenizer at 10,000psi.

In all cases, the pellet material was carried forward, while the soluble fraction was
45 discarded. The resultant solution was centrifuged in a GSA rotor for 1hr. at 12,500 rpm. The
pellet was resuspended in the same solution (without the DTT) using the same tissue
30 homogenizer probe at 2,000 rpm. After homogenizing for 5 minutes at 3000 rpm, the
volume was adjusted to 500ml with the same solution, and spun for 1hr. at 12,500 rpm.
50 The pellet was then resuspended as before, but this time the final volume was adjusted to

1.5L with the same solution prior to homogenizing for 5 minutes. After centrifuging at the same speed for 30 minutes, this procedure was repeated. The pellet was then resuspended into about 150ml of cold water, pooling the pellets from the six centrifuge tubes used in the GSA rotor. The pellet has homogenized for 5 minutes at 3,000 rpm, volume adjusted to 250ml with cold water, then spun for 30 minutes. Weight of the resultant pellet was 17.75g.

Summary: Lysis of bacterial pellet in KCl solution, followed by centrifugation in a GSA rotor was used to initially prepare the pellet. The same solution was then used an additional three times for resuspension/homogenization. A final water wash/homogenization was then performed to remove excess KCl and EDTA.

Solubilization of rHuAsp2L:

A ratio of 9-10ml/gram of pellet was utilized for solubilizing the rHuAsp2L from the pellet previously described. 17.75g of pellet was thawed, and 150ml of 8M guanidine HCl, 5mM β ME, 0.1% DEA, was added. 3M Tris was used to titrate the pH to 8.6. The pellet was initially resuspended into the guanidine solution using a 20mm tissue homogenizer probe at 1000 rpm. The mixture was then stirred at 4°C for 1 hour prior to centrifugation at 12,500rpm for 1 hour in GSA rotor. The resultant supernatant was then centrifuged for 30min at 40,000 x g in an SS-34 rotor. The final supernatant was then stored at -20°C, except for 50ml.

Immobilized Nickel Affinity Chromatography of Solubilized rHuAsp2L:

The following solutions were utilized:

- A) 6M Guanidine HCl, 0.1M NaP, pH 8.0, 0.01M Tris, 5mM β ME, 0.5mM Imidazole
- A') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl
- B') 6M Urea, 20mM NaP, pH 6.20, 50mM NaCl, 12mM Imidazole
- C') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl, 300mM Imidazole

Note: Buffers A' and C' were mixed at the appropriate ratios to give intermediate concentrations of Imidazole.

The 50ml of solubilized material was combined with 50ml of buffer A prior to adding to 100-125ml Qiagen Ni-NTA SuperFlow (pre-equilibrated with buffer A) in a 5 x 10cm Bio-Rad econo column. This was shaken gently overnight at 4°C in the cold room.

Chromatography Steps:

- 1) Drained the resultant flow through.
- 2) Washed with 50ml buffer A (collecting into flow through fraction)
- 3) Washed with 250ml buffer A (wash 1)
- 4) Washed with 250ml buffer A (wash 2)
- 5) Washed with 250ml buffer A'

- 5 6) Washed with 250ml buffer B'
7) Washed with 250ml buffer A'
8) Eluted with 250ml 75mM Imidazole
9) Eluted with 250ml 150mM Imidazole (150-1)
5 10) Eluted with 250ml 150mM Imidazole (150-2)
10 11) Eluted with 250ml 300mM Imidazole (300-1)
12) Eluted with 250ml 300mM Imidazole (300-2)
13) Eluted with 250ml 300mM Imidazole (300-3)

10 Chromatography Results:

15 The rHuAsp eluted at 75mM Imidazole through 300mM Imidazole. The 75mM fraction, as well as the first 150mM Imidazole (150-1) fraction contained contaminating proteins as visualized on Coomassie Blue stained gels. Therefore, fractions 150-2 and 300-1 will be utilized for refolding experiments since they contained the greatest amount of protein (see
20 15 Coomassie Blue stained gel).

Refolding Experiments of rHuAsp2L:

Experiment 1:

25 Forty ml of 150-2 was spiked with 1M DTT, 3M Tris, pH 7.4 and DEA to a final concentration of 6mM, 50mM, and 0.1% respectively. This was diluted suddenly (while
20 stirring) with 200ml of (4°C) cold 20mM NaP, pH 6.8, 150mM NaCl. This dilution gave a final Urea concentration of 1M. This solution remained clear, even if allowed to set open to the air at RT or at 4°C.

30 After setting open to the air for 4-5 hours at 4°C, this solution was then dialyzed overnight against 20mM NaP, pH 7.4, 150mM NaCl, 20% glycerol. This method effectively removes
25 the urea in the solution without precipitation of the protein.

Experiment 2:

35 Some of the 150-2 eluate was concentrated 2x on an Amicon Centriprep, 10,000 MWCO, then treated as in Experiment 1. This material also stayed in solution, with no visible
40 precipitation.

Experiment 3:

89ml of the 150-2 eluate was spiked with 1M DTT, 3M Tris, pH 7.4 and DEA to a final concentration of 6mM, 50mM, and 0.1% respectively. This was diluted suddenly (while stirring) with 445ml of (4°C) cold 20mM NaP, pH 6.8, 150mM NaCl. This solution appeared clear, with no apparent precipitation. The solution was removed to RT and stirred for 10 minutes prior to adding MEA to a final concentration of 0.1mM. This was stirred slowly at RT for 1hr. Cystamine and CuSO₄ were then added to final concentrations of 1mM and 10μM respectively. The solution was stirred slowly at RT for 10 minutes prior to being moved to the 4°C cold room and shaken slowly overnight, open to the air.

The following day, the solution (still clear, with no apparent precipitation) was centrifuged at 100,000 x g for 1 hour. Supernatants from multiple runs were pooled, and the bulk of the stabilized protein was dialyzed against 20mM NaP, pH 7.4, 150mM NaCl, 20% glycerol. After dialysis, the material was stored at -20°C.

Some (about 10ml) of the protein solution (still in 1M Urea) was saved back for biochemical analyses, and frozen at -20°C for storage.

Example 10. Expression of Hu-Asp2 and Derivatives in Insect Cells

Expression by baculovirus infection—The coding sequence of Hu-Asp2 and several derivatives were engineered for expression in insect cells using the PCR. For the full-length sequence, a 5'-sense oligonucleotide primer that modified the translation initiation site to fit the Kozak consensus sequence was paired with a 3'-antisense primer that contains the natural translation termination codon in the Hu-Asp2 sequence. PCR amplification of the pcDNA3.1(hygro)/Hu-Asp2 template (see Example 12). Two derivatives of Hu-Asp2 that delete the C-terminal transmembrane domain (SEQ ID No. 29 and No. 30) or delete the transmembrane domain and introduce a hexa-histidine tag at the C-terminus (SEQ ID No. 31 and No. 32) were also engineered using the PCR. The same 5'-sense oligonucleotide primer described above was paired with either a 3'-antisense primer that (1) introduced a translation termination codon after codon 453 (SEQ ID No. 3) or (2) incorporated a hexa-histidine tag followed by a translation termination codon in the PCR using pcDNA3.1(hygro)/Hu-Asp-2L as the template. In all cases, the PCR reactions were performed amplified for 15 cycles using PwoI DNA polymerase (Boehringer-Mannheim) as outlined by the supplier. The reaction products were digested to completion with *Bam*HI and *Not*I and ligated to *Bam*HI and *Not*I digested baculovirus transfer vector pVL1393 (Invitrogen). A portion of the ligations was used to transform competent *E. coli* DH5a cells

5 followed by antibiotic selection on LB-Amp. Plasmid DNA was prepared by standard alkaline lysis and banding in CsCl to yield the baculovirus transfer vectors pVL1393/Asp2, pVL1393/Asp2 Δ TM and pVL1393/Asp2 Δ TM(His)₆. Creation of recombinant baculoviruses and infection of sf9 insect cells was performed using standard methods.

10 5 *Expression by transfection*—Transient and stable expression of Hu-Asp2 Δ TM and Hu-Asp2 Δ TM(His)₆ in High 5 insect cells was performed using the insect expression vector pIZ/V5-His. The DNA inserts from the expression plasmids vectors pVL1393/Asp2, pVL1393/Asp2 Δ TM and pVL1393/Asp2 Δ TM(His)₆ were excised by double digestion with *Bam*HI and *Nor*I and subcloned into *Bam*HI and *Nor*I digested pIZ/V5-His using standard methods. The resulting expression plasmids, referred to as pIZ/Hu-Asp2 Δ TM and pIZ/Hu-Asp2 Δ TM(His)₆, were prepared as described above.

20 For transfection, High 5 insect cells were cultured in High Five serum free medium supplemented with 10 μ g/ml gentamycin at 27 °C in sealed flasks. Transfections were performed using High five cells, High five serum free media supplemented with 10 μ g/ml gentamycin, and InsectinPlus liposomes (Invitrogen, Carlsbad, CA) using standard methods.

25 For large scale transient transfections 1.2 x 10⁷ high five cells were plated in a 150 mm tissue culture dish and allowed to attach at room temperature for 15-30 minutes. During the attachment time the DNA/ liposome mixture was prepared by mixing 6 ml of serum free media, 60 μ g Asp2 Δ TM/pIZ (+/- His) DNA and 120 μ l of Insectin Plus and incubating at room temperature for 15 minutes. The plating media was removed from the dish of cells and replaced with the DNA/liposome mixture for 4 hours at room temperature with constant rocking at 2 rpm. An additional 6 ml of media was added to the dish prior to incubation for 4 days at 27 °C in a humid incubator. Four days post transfection the media was harvested, clarified by centrifugation at 500 x g, assayed for Asp2 expression by Western blotting. For stable expression, the cells were treated with 50 μ g/ml Zeocin and the surviving pool used to prepared clonal cells by limiting dilution followed by analysis of the expression level as noted above.

45 30 *Purification of Hu-Asp2 Δ TM and Hu-Asp2 Δ TM(His)₆*—Removal of the transmembrane segment from Hu-Asp2 resulted in the secretion of the polypeptide into the culture medium. Following protein production by either baculovirus infection or transfection, the conditioned medium was harvested, clarified by centrifugation, and dialyzed against Tris-HCl (pH 8.0). This material was then purified by successive

5 chromatography by anion exchange (Tris-HCl, pH 8.0) followed by cation exchange
chromatography (Acetate buffer at pH 4.5) using NaCl gradients. The elution profile was
monitored by (1) Western blot analysis and (2) by activity assay using the peptide substrate
described in Example 12. For the Hu-Asp2ΔTM(His)₆, the conditioned medium was
10 5 dialyzed against Tris buffer (pH 8.0) and purified by sequential chromatography on IMAC
resin followed by anion exchange chromatography.

15 Sequence analysis of the purified Hu-Asp2ΔTM(His)₆ protein revealed that the signal
peptide had been cleaved [TQHGIRLPLR].

10 **Example 11. Expression of Hu-Asp2 in CHO cells**

20 *Heterologous expression of Hu-Asp-2L in CHO-K1 cells*—The entire coding sequence of
Hu-Asp2 was cloned into the mammalian expression vector pcDNA3.1(+)-Hygro
15 (Invitrogen, Carlsbad, CA) which contains the CMV immediate early promoter and bGH
polyadenylation signal to drive over expression. The expression plasmid,
25 pcDNA3.1(+)-Hygro/Hu-Asp2, was prepared by alkaline lysis and banding in CsCl and
completely sequenced on both strands to verify the integrity of the coding sequence.

30 20 Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the ATCC. The
cells were maintained in monolayer cultures in α-MEM containing 10% FCS at 37°C in 5%
35 CO₂. Two 100 mm dishes of CHO-K1 cells (60% confluent) were transfected with
pcDNA3.1(+)-Hygro alone (mock) or pcDNA3.1(+)-Hygro/Hu-Asp2 using the cationic
liposome DOTAP as recommended by the supplier. The cells were treated with the plasmid
40 25 DNA/liposome mixtures for 15 hr and then the medium replaced with growth medium
containing 500 Units/ml hygromycin B. In the case of pcDNA3.1(+)-Hygro/Hu-Asp2
45 transfected CHO-K1 cells, individual hygromycin B-resistant cells were cloned by limiting
dilution. Following clonal expansion of the individual cell lines, expression of Hu-Asp2
protein was accessed by Western blot analysis using a polyclonal rabbit antiserum raised
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5 against recombinant Hu-Asp2 prepared by expression in *E. coli*. Near confluent dishes of
each cell line were harvested by scraping into PBS and the cells recovered by
centrifugation. The cell pellets were resuspended in cold lysis buffer (25 mM Tris-HCl
10 (8.0)/5 mM EDTA) containing protease inhibitors and the cells lysed by sonication. The
soluble and membrane fractions were separated by centrifugation (105,000 x g, 60 min) and
normalized amounts of protein from each fraction were then separated by SDS-PAGE.
15 Following electrotransfer of the separated polypeptides to PVDF membranes, Hu-Asp-2L
protein was detected using rabbit anti-Hu-Asp2 antiserum (1/1000 dilution) and the
antibody-antigen complexes were visualized using alkaline phosphatase conjugated goat
20 anti-rabbit antibodies (1/2500). A specific immunoreactive protein with an apparent Mr
value of 65 kDa was detected in pcDNA3.1(+)/Hygro/Hu-Asp2 transfected cells and not
25 mock-transfected cells. Also, the Hu-Asp2 polypeptide was only detected in the membrane
fraction, consistent with the presence of a signal peptide and single transmembrane domain
in the predicted sequence. Based on this analysis, clone #5 had the highest expression level
30 of Hu-Asp2 protein and this production cell lines was scaled up to provide material for
purification.

35 *Purification of recombinant Hu-Asp-2L from CHO-K1/Hu-Asp2 clone #5*—In a
typical purification, clone #5 cell pellets derived from 20 150 mm dishes of confluent cells,
were used as the starting material. The cell pellets were resuspended in 50 ml cold lysis
40 buffer as described above. The cells were lysed by polytron homogenization (2 x 20 sec)
and the lysate centrifuged at 338,000 x g for 20 minutes. The membrane pellet was then
45 resuspended in 20 ml of cold lysis buffer containing 50 mM β -octylglucoside followed by
rocking at 4°C for 1hr. The detergent extract was clarified by centrifugation at 338,000 x g
for 20 minutes and the supernatant taken for further analysis.

5 The β -octylglucoside extract was applied to a Mono Q anion exchange column that
was previously equilibrated with 25 mM Tris-HCl (pH 8.0)/50 mM β -octylglucoside.
Following sample application, the column was eluted with a linear gradient of increasing
10 NaCl concentration (0-1.0 M over 30 minutes) and individual fractions assayed by Western
5 blot analysis and for β -secretase activity (see below). Fractions containing both Hu_Asp-
2L immunoreactivity and β -secretase activity were pooled and dialyzed against 25 mM
15 NaOAc (pH 4.5)/50 mM β -octylglucoside. Following dialysis, precipitated material was
removed by centrifugation and the soluble material chromatographed on a MonoS cation
20 exchange column that was previously equilibrated in 25 mM NaOAc (pH 4.5)/ 50 mM β -
10 octylglucoside. The column was eluted using a linear gradient of increasing NaCl
concentration (0-1.0 M over 30 minutes) and individual fractions assayed by Western blot
25 analysis and for β -secretase activity. Fractions containing both Hu-Asp2 immunoreactivity
and β -secretase activity were combined and determined to be >90% pure by SDS-
30 PAGE/Coomassie Blue staining.

15 **Example 12. Assay of Hu-Asp2 β -secretase activity using peptide substrates**

β -secretase assay— β -secretase activity was measured by quantifying the hydrolysis of a
35 synthetic peptide containing the APP Swedish mutation by RP-HPLC with UV detection.
Each reaction contained 50 mM Na-MES (pH 5.5), 1% β -octylglucoside, peptide substrate
(SEVNLDAEFR, 70 μ M) and enzyme (1-5 μ g protein). Reactions were incubated at 37 °C
40 for various times and the reaction products were resolved by RP-HPLC using a linear
20 gradient from 0-70 B over 30 minutes (A=0.1% TFA in water,
45 B=).1%TFA/10%water/90%AcCN). The elution profile was monitored by absorbance at
214 nm. In preliminary experiments, the two product peaks which eluted before the intact
peptide substrate, were confirmed to have the sequence DAEFR and SEVNL using both
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5 Edman sequencing and MADLI-TOF mass spectrometry. Percent hydrolysis of the peptide
substrate was calculated by comparing the integrated peak areas for the two product
10 peptides and the starting material derived from the absorbance at 214 nm. The specificity
of the protease cleavage reaction was determined by performing the β -secretase assay in the
5 presence of a cocktail of protease inhibitors (8 μ M pepstatin A, 10 μ M leupeptin, 10 μ M
E64, and 5 mM EDTA).

15 An alternative β -secretase assay utilizes internally quenched fluorescent substrates
to monitor enzyme activity using fluorescence spectroscopy in a single sample or multiwell
format. Each reaction contained 50 mM Na-MES (pH 5.5), peptide substrate MCA-
20 EVKMDAEF[K-DNP] (BioSource International) (50 μ M) and purified Hu-Asp-2 enzyme.
These components were equilibrated to 37 °C for various times and the reaction initiated by
addition of substrate. Excitation was performed at 330 nm and the reaction kinetics were
25 monitored by measuring the fluorescence emission at 390 nm. To detect compounds that
modulate Hu-Asp-2 activity, the test compounds were added during the preincubation phase
15 of the reaction and the kinetics of the reaction monitored as described above. Activators are
scored as compounds that increase the rate of appearance of fluorescence while inhibitors
decrease the rate of appearance of fluorescence.
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It will be clear that the invention may be practiced otherwise than as particularly
described in the foregoing description and examples.

20 Numerous modifications and variations of the present invention are possible in light of the
above teachings and, therefore, are within the scope of the invention.

The entire disclosure of all publications cited herein are hereby incorporated by reference.

Claims

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What is claimed is:

1. Any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta (β) secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code for the peptide DTG, where the first nucleic acid of the first special set of nucleic acids is, the first special nucleic acid, and where the second set of nucleic acids code for either the peptide DSG or DTG, where the last nucleic acid of the second set of nucleic acids is the last special nucleic acid, with the proviso that the nucleic acids disclosed in SEQ ID NO. 1 and SEQ. ID NO. 5 are not included.
2. The nucleic acid polynucleotide of claim 1 where the two sets of nucleic acids are separated by nucleic acids that code for about 125 to 222 amino acid positions, which may be any amino acids.
3. The nucleic acid polynucleotide of claim 2 that code for about 150 to 172 amino acid positions, which may be any amino acids.
4. The nucleic acid polynucleotide of claim that code for about 172 amino acid positions, which may be any amino acids.
5. The nucleic acid polynucleotide of claim 4 where the nucleotides are described in SEQ. ID. NO. 3
6. The nucleic acid polynucleotide of claim 2 where the two sets of nucleic acids are separated by nucleic acids that code for about 150 to 196 amino acid positions.
7. The nucleic acid polynucleotide of claim 6 where the two sets of nucleotides are separated by nucleic acids that code for about 196 amino acids (positions).

- 5 8. The nucleic acid polynucleotide of claim 7 where the two sets of nucleic acids are separated by the same nucleic acid sequences that separate the same set of special nucleic acids in SEQ. ID. NO. 5.
- 10 9. The nucleic acid polynucleotide of claim 4 where the two sets of nucleic acids are separated by nucleic acids that code for about 150 to 190, amino acid (positions).
- 15 10. The nucleic acid polynucleotide of claim 9 where the two sets of nucleotides are separated by nucleic acids that code for about 190 amino acids (positions).
- 10 11. The nucleic acid polynucleotide of claim 10 where the two sets of nucleotides are separated by the same nucleic acid sequences that separate the same set of special nucleotides in SEQ. ID. NO. 1.
- 20 12. Claims 1-11 where the first nucleic acid of the first special set of amino acids, that is, the first special nucleic acid, is operably linked to any codon where the nucleic acids of that codon codes for any peptide comprising from 1 to 10,000 amino acid (positions).
- 25 13. The nucleic acid polynucleotide of claims 1-12 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any any reporter proteins or proteins which facilitate purification.
- 30 14. The nucleic acid polynucleotide of claims 1-13 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.
- 35 15. Claims 1-14 where the last nucleic acid of the second set of special amino acids, that is, the last special nucleic acid, is operably linked to nucleic acid polymers that code for any peptide comprising any amino acids from 1 to 10,000 amino acids.

- 5 16. Claims 1-15 where the last special nucleic acid is operably linked to any codon linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification.
- 10 17. The nucleic acid polynucleotide of claims 1-16 where the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.
- 15 18. * Any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code for DTG, where the first nucleic acid of the first special set of nucleic acids is, the first special nucleic acid, and where the second set of nucleic acids code for either DSG or DTG, where the last nucleic acid of the second set of special nucleic acids is the last special nucleic acid, where the first special nucleic acid is operably linked to nucleic acids that code for any number of amino acids from zero to 81 amino acids and where each of those codons may code for any amino acid.
- 20 19. The nucleic acid polynucleotide of claim 18 , where the first special nucleic acid is operably linked to nucleic acids that code for any number of from 64 to 77 amino acids where each codon may code for any amino acid.
- 25 20. The nucleic acid polynucleotide of claim 19 , where the first special nucleic acid is operably linked to nucleic acids that code for about 71 amino acids peptide.
- 30 21. The nucleic acid polynucleotide of claim 20, where the first special nucleic acid is operably linked to 71 amino acid peptide and where the first of those 71 amino acids is the amino acid T.

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22. The nucleic acid polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 71 amino acids, see Example 10, beginning from the DTG site and including the nucleotides from that code for 71 amino acids).
23. The nucleic acid polynucleotide of claim 22, where the complete polynucleotide comprises identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 71 amino acids, see Example 10, beginning from the DTG site and including the nucleotides from that code for 71 amino acids).
24. The nucleic acid polynucleotide of claim 18, where the first special nucleic acid is operably linked to nucleic acids that code for any number of from about 30 to 54 amino acids where each codon may code for any amino acid.
25. The nucleic acid polynucleotide of claim 20, where the first special nucleic acid is operably linked to 47 codons where the first those 35 or 47 amino acids is the amino acid E or G.
26. The nucleic acid polynucleotide of claim 21, where the polynucleotide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to that portion of the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 35 or 47 amino acids, see Example 11 for the 47 example, beginning from the DTG site and including the nucleotides from that code for the previous 35 or 47 amino acids before the DTG site).

- 5 27. The nucleic acid polynucleotide of claim 22, where the polynucleotide comprises identical to the same corresponding amino acids in SEQ. ID. NO. 3, that is, identical to the sequences in SEQ. ID. NO. 3 including the sequences from both the first and or the second special nucleic acids, toward the N-Terminal, through and including 35 or 47 amino acids, see Example 11 for the 47 example, beginning from the DTG site and including the nucleotides from that code for the previous 35 or 47 amino acids before the DTG site).
- 10 28. * Any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta (β) secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code for the peptide DTG, where the first nucleic acid of the first special set of amino acids is, the first special nucleic acid, and where the second set of special nucleic acids code for either the peptide DSG or DTG, where the last nucleic acid of the second set of special nucleic acids, the last special nucleic acid, is operably linked to nucleic acids that code for any number of codons from 50 to 170 codons.
- 15 29. The nucleic acid polynucleotide of claim 29 where the last special nucleic acid is operably linked to nucleic acids comprising from 100 to 170 codons.
- 20 30. The nucleic acid polynucleotide of claim 30 where the last special nucleic acid is operably linked to nucleic acids comprising from 142 to 163 codons.
- 25 31. The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 142 codons.
- 30 32. The nucleic acid polynucleotide of claim 32 where the polynucleotide comprises a sequence that is at least 95% identical to SEQ. ID. # (Example 9 or 10).

- 5 33. The nucleic acid polynucleotide of claim 33, where the complete polynucleotide comprises SEQ. ID. # (Example 9 or 10).
- 10 34. The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 163 codons.
- 15 35. The nucleic acid polynucleotide of claim 35 where the polynucleotide comprises a sequence that is at least 95% identical to SEQ. ID. # (Example 9 or 10).
- 20 36. The nucleic acid polynucleotide of claim 36, where the complete polynucleotide comprises SEQ. ID. # (Example 9 or 10).
- 25 37. The nucleic acid polynucleotide of claim 31 where the last special nucleic acid is operably linked to nucleic acids comprising about 170 codons.
- 30 38. Claims 1-38 where the second set of special nucleic acids code for the peptide DSG, and optionally the first set of nucleic acid polynucleotide is operably linked to a peptide purification tag.
- 35 39. Claims 1-39 where the nucleic acid polynucleotide is operably linked to a peptide purification tag which is six histidine.
- 40 40. Claims 1-40 where the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a second polynucleotide, where both first and second polynucleotides have at least 50 codons.
- 45 41. Claims 1-40 where the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a second polynucleotide, where both first and second polynucleotides have at least 50 codons where both said polynucleotides are in the same solution.
- 50 42. A vector which contains a polynucleotide described in claims 1-42.

- 5 43. A cell or cell line which contains a polynucleotide described in claims 1-42.
- 10 44. Any isolated or purified peptide or protein comprising an amino acid polymer that is
5 a protease capable of cleaving the beta (β) secretase cleavage site of APP that
contains two or more sets of special amino acids, where the special amino acids are
separated by about 100 to 300 amino acid positions, where each amino acid
position can be any amino acid, where the first set of special amino acids consists of
15 the peptide DTG, where the first amino acid of the first special set of amino acids is,
the first special amino acid, where the second set of amino acids is selected from the
peptide comprising either DSG or DTG, where the last amino acid of the second set
10 of special amino acids is the last special amino acid, with the proviso that the
proteases disclosed in SEQ ID NO. 2 and SEQ. ID NO. 6 are not included.
- 20 45. The amino acid polypeptide of claim 45 where the two sets of amino acids are
15 separated by about 125 to 222 amino acid positions where in each position it may be
any amino acid.
- 30 46. The amino acid polypeptide of claim 46 where the two sets of amino acids are
separated by about 150 to 172 amino acids.
- 20 47. The amino acid polypeptide of claim 47 where the two sets of amino acids are
separated by about 172 amino acids.
- 35 48. The amino acid polypeptide of claim 48 where the protease is described in SEQ. ID.
25 NO. 4
- 40 49. The amino acid polypeptide of claim 46 where the two sets of amino acids are
separated by about 150 to 196 amino acids.
- 45 50. The amino acid polypeptide of claim 50 where the two sets of amino acids are
30 separated by about 196 amino acids.

- 5 51. The amino acid polypeptide of claim 51 where the two sets of amino acids are separated by the same amino acid sequences that separate the same set of special amino acids in SEQ. ID. NO. 6.
- 10 52. The amino acid polypeptide of claim 46 where the two sets of amino acids are separated by about 150 to 190, amino acids.
- 15 53. The amino acid polypeptide of claim 53 where the two sets of nucleotides are separated by about 190 amino acids.
- 20 54. The amino acid polypeptide of claim 54 where the two sets of nucleotides are separated by the same amino acid sequences that separate the same set of special amino acids in SEQ. ID. NO. 2.
- 25 55. Claims 45-55 where the first amino acid of the first special set of amino acids, that is, the first special amino acid, is operably linked to any peptide comprising from 1 to 10,000 amino acids.
- 30 56. The amino acid polypeptide of claims 45-56 where the first special amino acid is operably linked to any peptide selected from the group consisting of: any any reporter proteins or proteins which facilitate purification.
- 35 57. The amino acid polypeptide of claims 45-57 where the first special amino acid is operably linked to any peptide selected from the group consisting of:
25 immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.
- 40 58. Claims 45-58, where the last amino acid of the second set of special amino acids, that is, the last special amino acid, is operably linked to any peptide comprising any
45 amino acids from 1 to 10,000 amino acids.
- 50
- 55

5 59. Claims 45-59 where the last special amino acid is operably linked any peptide selected from the group consisting of any reporter proteins or proteins which facilitate purification.

10 5 60. The amino acid polypeptide of claims 45-60 where the first special amino acid is operably linked to any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathion S transfection, Green Fluorescent protein, and ubiquitin.

15 10 61. * Any isolated or purified peptide or protein comprising an amino acid polypeptide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 100 to 300 amino acid positions, where each amino acid in each position can be any amino acid, where the first set of special amino acids consists of the amino acids DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, D, and where the second set of amino acids is either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, G, where the first special amino acid is operably linked to amino acids that code for any number of amino acids from zero to 81 amino acid positions where in each position it may be any amino acid.

35 62. The amino acid polypeptide of claim 62, where the first special amino acid is operably linked to a peptide from about 30 to 77 amino acids positions where each amino acid position may be any amino acid.

40 63. The amino acid polypeptide of claim 63, where the first special amino acid is operably linked to a peptide of 35, 47, 71, or 77 amino acids.

45 30 64. The amino acid polypeptide of claim 63, where the first special amino acid is operably linked to the same corresponding peptides from SEQ. ID. NO. 3 that are 35, 47, 71, or 77 peptides in length, beginning counting with the amino acids on the first special sequence, DTG, towards the N-terminal of SEQ. ID. NO. 3.

- 5 65. The amino acid polypeptide of claim 65, where the polypeptide comprises a
sequence that is at least 95% identical to the same corresponding amino acids in
SEQ. ID. NO. 4, that is, identical to that portion of the sequences in SEQ.ID. NO. 4,
10 5 including all the sequences from both the first and or the second special nucleic
acids, toward the N- terminal, through and including 71, 47, 35 amino acids before
the first special amino acids. (Examples 10 and 11).
- 15 66. The amino acid polypeptide of claim 65, where the complete polypeptide comprises
10 the peptide of 71 amino acids, where the first of the amino acid is T and the second
is Q.
- 20 67. The amino acid polypeptide of claim 62, where the first special amino acid is
operably linked to any number of from 40 to 54 amino acids (positions) where each
15 amino acid position may be any amino acid.
- 25 68. The amino acid polypeptide of claim 68, where the first special amino acid is
operably linked to amino acids that code for a peptide of 47 amino acids.
- 30 69. The amino acid polypeptide of claim 69, where the first special amino acid is
operably linked to a 47 amino acid peptide where the first those 47 amino acids is
35 the amino acid E.
- 40 70. The amino acid polypeptide of claim 70, where the polypeptide comprises a
25 sequence that is at least 95% identical to SEQ. ID. # (Example 10).
- 45 71. The amino acid polypeptide of claim 71, where the complete polypeptide comprises
SEQ. ID. # (Example 10).
- 50 72. * Any isolated or purified amino acid polypeptide that is a protease capable of
cleaving the beta (β) secretase cleavage site of APP that contains two or more sets
of special amino acids, where the special amino acids are separated by about 100 to
300 amino acid positions, where each amino acid in each position can be any amino

acid, where the first set of special amino acids consists of the amino acids that code for DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, D, and where the second set of amino acids are either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, G, which is operably linked to any number of amino acids from 50 to 170 amino acids, which may be any amino acids.

73. The amino acid polypeptide of claim 73 where the last special amino acid is operably linked to a peptide of about 100 to 170 amino acids.

74. The amino acid polypeptide of claim 74 where the last special amino acid is operably linked to a peptide of about 142 to 163 amino acids.

75. The amino acid polypeptide of claim 75 where the last special amino acid is operably linked to a peptide of about about 142 amino acids.

76. The amino acid polypeptide of claim 76 where the polypeptide comprises a sequence that is at least 95% identical to SEQ. ID. # (Example 9 or 10).

77. The amino acid polypeptide of claim 75 where the last special amino acid is operably linked to a peptide of about 163 amino acids.

78. The amino acid polypeptide of claim 79 where the polypeptide comprises a sequence that is at least 95% identical to SEQ. ID. # (Example 9 or 10).

79. The amino acid polypeptide of claim 79, where the complete polypeptide comprises SEQ. ID. # (Example 9 or 10).

80. The amino acid polypeptide of claim 74 where the last special amino acid is operably linked to a peptide of about 170 amino acids.

81. Claim 46-81 where the second set of special amino acids is comprised of the peptide with the amino acid sequence DSG.

- 5 82. Claims 45-82 where the amino acid polypeptide is operably linked to a peptide purification tag.
- 10 83. Claims 45-83 where the amino acid polypeptide is operably linked to a peptide purification tag which is six histidine.
- 15 84. Claims 45-84 where the first set of special amino acids are on one polypeptide and the second set of special amino acids are on a second polypeptide, where both first and second polypeptide have at least 50 amino acids, which may be any amino acids.
- 20 85. Claims 45-84 where the first set of special amino acids are on one polypeptide and the second set of special amino acids are on a second polypeptide, where both first and second polypeptides have at least 50 amino acids where both said polypeptides are in the same vessel.
- 25 86. A vector which contains a polypeptide described in claims 45-86.
- 30 87. A cell or cell line which contains a polynucleotide described in claims 45-87.
- 35 88. The process of making any of the polynucleotides, vectors, or cells of claims 1-44
- 40 89. The process of making any of the polypeptides, vectors or cells of claims 45-88
- 45 90. Any of the polynucleotides, polypeptides, vectors, cells or cell lines described in claims 1-88 made from the processes described in claims 89 and 90.
- 50 91. * An isolated nucleic acid molecule comprising a polynucleotide, said polynucleotide encoding a Hu-Asp polypeptide and having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

5 (a) a nucleotide sequence encoding a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), wherein said Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) polypeptides have the complete amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID No:6, respectively; and

10 5 (b) a nucleotide sequence complementary to the nucleotide sequence of (a).

15 92. The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is Hu-Asp1, and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID NO:1.

20 93. The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is Hu-Asp2(a), and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID NO:4.

25 94. The nucleic acid molecule of claim 92, wherein said Hu-Asp polypeptide is Hu-Asp2(b), and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID NO:5.

30 95. An isolated nucleic acid molecule comprising polynucleotide which hybridizes under stringent conditions to a polynucleotide having the nucleotide sequence in (a) or (b) of claim 92.

35 96. A vector comprising the nucleic acid molecule of claim 96.

40 97. The vector of claim 97, wherein said nucleic acid molecule is operably linked to a promoter for the expression of a Hu-Asp polypeptide.

45 98. The vector of claim 97, wherein said Hu-Asp polypeptide is Hu-Asp1.

50 99. The vector of claim 97, wherein said Hu-Asp polypeptide is Hu-Asp2(a).

100. The vector of claim 97, wherein said Hu-Asp polypeptide is Hu-Asp2(b).

5 101. A host cell comprising the vector of claim 98.

10 102. A method of obtaining a Hu-Asp polypeptide comprising culturing the host cell of
5 claim 102 and isolating said Hu-Asp polypeptide.

15 103. An isolated Hu-Asp1 polypeptide comprising an amino acid sequence at least 95%
identical to a sequence comprising the amino acid sequence of SEQ ID NO:2.

20 104. An isolated Hu-Asp2(a) polypeptide comprising an amino acid sequence at least
95% identical to a sequence comprising the amino acid sequence of SEQ ID NO:4.

25 105. An isolated Hu-Asp2(a) polypeptide comprising an amino acid sequence at least
95% identical to a sequence comprising the amino acid sequence of SEQ ID NO:8.

30 106. An isolated antibody that binds specifically to the Hu-Asp polypeptide of any of
claims 104-107.
sequence comprising the amino acid sequence of SEQ ID NO:8.

35 107. An isolated antibody that binds specifically to the Hu-Asp polypeptide of any of
claims 104-107.

40 108. * A method to identify a cell that can be used to screen for inhibitors of β
secretase activity comprising:

- 45 25 a) identifying a cell that expresses a protease capable of cleaving APP at the β
secretase site,
comprising:
i) collect the cells or the supernatant from the cells to be identified
ii) measure the production of a critical peptide, where the critical
30 peptide is selected from the group consisting of either the APP C-
terminal peptide or soluble APP,
iii) select the cells which produce the critical peptide.

5 109. The method of claim 108 where the cells are collected and the critical peptide is the APP C-terminal peptide created as a result of the β secretase cleavage.

10 110. The method of claim 108 where the supernatant is collected and the critical peptide is soluble APP where the soluble APP has a C-terminal created by β secretase cleavage.

15 111. The method of claim 108 where the cells contain any of the nucleic acids or polypeptides of claims 1-86 and where the cells are shown to cleave the β secretase site of any peptide having the following peptide structure, P2, P1, P1', P2', where P2 is K or N, where P1 is M or L, where P1' is D, where P2' is A.

20 112. The method of claim 111 where P2 is K and P1 is M.

25 113. The method of claim 112 where P2 is N and P1 is L.

30 114. * Any bacterial cell comprising any nucleic acids or peptides in claims 1-86 and 92-107.

35 115. A bacterial cell of claim 114 where the bacteria is *E. coli*.

40 116. Any eukaryotic cell comprising any nucleic acids or polypeptides in claims 1-86 and 92-107.

45 117. * Any insect cell comprising any of the nucleic acids or polypeptides in claims 1-86 and 92-107.

50 118. A insect cell of claim 117 where the insect is sf9, or High 5.

55 119. A insect cell of claim 100 where the insect cell is High 5.

120. A mammalian cell comprising any of the nucleic acids or polypeptides in claims 1-86 and 92-107.

5 121 A mammalian cell of claim 120 where the mammalian cell is selected from the group consisting of, human, rodent, lagomorph, and primate.

10 122 A mammalian cell of claim 121 where the mammalian cell is selected from the group consisting of human cell.

15 123 A mammalian cell of claim 122 where the human cell is selected from the group comprising HEK293, and IMR-32.

20 124 A mammalian cell of claim 121 where the cell is a primate cell.

25 125 A primate cell of claim 124 where the primate cell is a COS-7 cell.

30 126 A mammalian cell of claim 121 where cell is selected from a rodent cells.

35 127 A rodent cell of claim 126 selected from, CHO-K1, Neuro-2A, 3T3 cells.

40 128 A yeast cell of claim 115.

45 129 An avian cell of claim 115.

50 130. * Any isoform of APP where the last two carboxy terminus amino acids of that isoform are both lysine residues.

55 131 The isoform of APP from claim 130 comprising the isoform known as APP695 modified so that its last two having two lysine residues as its last two carboxy terminus amino acids.

132 The isoform of claim 131 comprising SEQ. ID. 16.

133 The isoform variant of claim 130 comprising SEQ. ID. NO. 18, and 20.

5 134 Any eukaryotic cell line, comprising nucleic acids or polypeptides of claim 130-133.

10 135 Any cell line of claim 134 that is a mammalian cell line (HEK293, Neuro2a, are preferred plus any others.)

15 136 A method for identifying inhibitors of an enzyme that cleaves the beta secretase cleavable site of APP comprising:

- 10 a) culturing cells in a culture medium under conditions in which the enzyme causes processing of APP and release of amyloid beta-peptide into the medium and causes the accumulation of CTF99 fragments of APP in cell lysates,
- 20 b) exposing the cultured cells to a test compound; and specifically determining whether the test compound inhibits the function of the enzyme by
- 25 measuring the amount of amyloid beta-peptide released into the medium and or the amount of CTF99 fragments of APP in cell lysates;
- 30 c) identifying test compounds diminishing the amount of soluble amyloid beta peptide present in the culture medium and diminution of CTF99 fragments of APP in cell lysates as Asp2 inhibitors.

20

35 137 The method of claim 136 wherein the cultured cells are a human, rodent or insect cell line.

40 138 The method of claim 137 wherein the human or rodent cell line exhibits β secretase activity in which processing of APP occurs with release of amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates.

45 139. A method as in claim 138 wherein the human or rodent cell line treated with the antisense oligomers directed against the enzyme that exhibits β secretase activity, reduces

50 30 release of soluble amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates.

50

55

5 140. A method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

10 (a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

(b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

15 whereby a lower level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has decreased the activity of said Hu-Asp polypeptide..

20 141. The nucleic acids, peptides, proteins, vectors, cells and cell lines, and assays described herein.

FIGURE 1 (1)

ATGGGCGCACTGGCCCCGGGCGCTGCTGCTGCTCTGCTGGCCCCAGTGGCTCCTGGCGGCC
M G A L A R A L L L P L L A Q W L L R A
CCCCGGAGCTGGCCCCCGCGCCCTTCAGCTGCCCCCTCCGGGTGGCCGCGCCACGAAC
A P E L A P A P F T L P L R V A A A T N
CGCGTAGTTGCGCCACCCCGGGACCCGGGACCCCTGCGGAGCGCCACGCGGACGGCTTG
R V V A P T P G P G T P A E R H A D G L
GCGCTCGCCCTGGAGCCTGCGCTGCGCTCCCCCGCGGGCGCCCAACTTCTTGGCCATG
A L A L E P A L A S P A G A A N F L A M
GTAGACAACCTGCAGGGGACTCTGCGCGCGGCTACTACCTGGAGATGCTGATCGGGACC
V D N L Q G D S G R G Y Y L E M L I G T
CCCCCGCAGAAGCTACAGATTCTCGTTGACACTGGAAGCAGTAACTTTGCCGTGGCAGGA
P P Q K L Q I L V D T G S S N F A V A G
ACCCCGCACTCCTACATAGACACGTACTTTGACACAGAGAGGTCTAGCACATACCGCTCC
T P H S Y I D T Y F D T E R S S T Y R S
AAGGGCTTTGACGTCACAGTGAAGTACACACAAGGAAGCTGGACGGGCTTCGTTGGGGAA
K G F D V T V K Y T Q G S W T G F V G E
GACCTCGTACCATCCCCAAGGCTTCAATACTTCTTTCTTGTCAACATTGCCACTATT
D L V T I P K G F N T S F L V N I A T I
TTTGAATCAGAGAATTTCTTTTTCCTGGGATTAAATGGAATGGAATACTTGGCCTAGCT
F E S E N F F L P G I K W N G I L G L A
TATGCCACACTTGCCAAGCCATCAAGTTCTCTGGAGACCTTCTTCGACTCCCTGGTGACA
Y A T L A K P S S S L E T F F D S L V T
CAAGCAAACATCCCCAAGCTTTTCTCCATGCAGATGTGTGGAGCCGGCTTGCCCGTTGCT
Q A N I P N V F S M Q M C G A G L P V A
GGATCTGGGACCAACGGAGGTAGTCTTGTCTTGGGTGGAATTGAACCAAGTTTGTATAAA
G S G T N G G S L V L G G I E P S L Y K
GGAGACATCTGGTATACCCCTATTAAGGAAGAGTGGTACTACCAGATAGAAAATCTGAAA
G D I W Y T P I K E E W Y Y Q I E I L K
TTGGAAATTGGAGGCCAAAGCCTTAATCTGGACTGCAGAGAGTATAACGCAGACAAGGCC
L E I G G Q S L N L D C R E Y N A D K A
ATCGTGGACAGTGGCACCCAGCTGCTGCGCCTGCCCCAGAAGGTGTTTGATGCGGTGCTG
I V D S G T T L L R L P Q K V F D A V V
GAAGCTGTGGCCCCCGCATCTCTGATTCCAGAATTCTCTGATGGTTTCTGGACTGGGTCC
E A V A R A S L I P E F S D G F W T G S
CAGCTGGCGTGCTGGACGAATTCCGAAACACCTTGGTCTTACTTCCCTAAAATCTCCATC
Q L A C W T N S E T P W S Y F P K I S I
TACCTGAGAGATGAGAACTCCAGCAGGTCAATCCGTATCACAATCCTGCCTCAGCTTTAC
Y L R D E N S S R S F R I T I L P Q L Y
ATTCAGCCCATGATGGGGGCGGCTGAATTATGAATGTTACCGATTCCGGCATTTCGCCA
I Q P M M G A G L N Y E C Y R F G I S P
TCCACAAATGCGCTGGTGATCGGTGCCACGGTGATGGAGGGCTTCTACGTCATCTTCGAC
S T N A L V I G A T V M E C F Y V I F D
AGAGCCCAAGAGGGTGGGCTTTCGAGCGAGCCCTGTGCAGAAATTGCAGGTGCTGCA

FIGURE 1 (2)

R A Q K R V G F A A S P C A E I A G A A
GTGTCGAAATTTCGGGCCTTTCTCAACAGAGGATGTAGCCAGCAACTGTGTCCCGCT
V S E I S G P F S T E D V A S N C V P A
CAGTCTTTGAGCGAGCCCATTTGTGGATTGTGTCCTATGCGCTCATGAGCGTCTGTGA
Q S L S E P I L W I V S Y A L M S V C G
GCCATCCTCCTTGTCTTAATCGTCCTGCTGCTGCTGCCGTTCCGGTGTGAGCGTCGCCCC
A I L L V L I V L L L L P F R C Q R R P
CGTGACCCTGAGGTCGTCAATGATGAGTCCTCTCTGGTCAGACATCGCTGGAATGAATA
R D P E V V N D E S S L V R H R W K
GCCAGGCCTGACCTCAAGCAACCATGAACTCAGCTATTAAGAAATCACATTTCCAGGGC
AGCAGCCGGGATCGATGGTGGCGCTTTCTCCTGTGCCACCCGCTTCAATCTCTGTCT
GCTCCCAGATGCCTTCTAGATTCACTGCTTTTGATTCTTGATTTTCAAGCTTTCAAATC
CTCCCTACTTCCAAGAAAAATAATTAATAAAAAAACTTCATTCTAAACCAAAAAA
AAAA

FIGURE 2 (1)

ATGGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGCGGGAGTGTGCCTGCCAC
M A Q A L P W L L L W M G A G V L P A H
GGCACCAGCACGGCATCCGGCTGCCCTGCGCAGCGGCCTGGGGGGCGCCCCCTGGGG
G T Q H G I R L P L R S G L G G A P L G
CTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGCGGAGGGGCGAGCTTT
L R L P R E T D E E P E E P G R R G S F
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC
V E M V D N L R G K S G Q G Y Y V E M T
GTGGGCAGCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACCTTTGCA
V G S P P Q T L N I L V D T G S S N F A
GTGGGTGCTGCCCCCACCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V G A A P H P F L H R Y Y Q R Q L S S T
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAG
Y R D L R K G V Y V P Y T Q G K W E G E
CTGGGCACCGACCTGTAAGCATCCCCCATGGCCCCAACGTCAGTGTGCGTGCCAACATT
L G T D L V S I P H G P N V T V R A N I
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAAGTGGGAAGGCATCCTG
A A I T E S D K F F I N G S N W E G I L
GGGCTGGCCTATGCTGAGATTGCCAGGCTTTGTGGTGTGGCTTCCCCCTCAACAGTCT
G L A Y A E I A R L C G A G F P L N Q S
GAAGTGCTGGCCTCTGTGCGAGGGAGCATGATCATTGGAGGTATCGACCACTCGCTGTAC
E V L A S V G G S M I I G G I D H S L Y
ACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTATGAGGTGATCATTGTG
T G S L W Y T P I R R E W Y Y E V I I V
CGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAGTACAACCTATGACAAG
R V E I N G Q D L K M D C K E Y N Y D K
AGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAAGTGTGTTGAAGCTGCA
S I V D S G T T N L R L P K K V F E A A
GTCAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCTGATGGTTTCTGGCTAGGA
V K S I K A A S S T E K F P D G F W L G
GAGCAGCTGGTGTGCTGGCAAGCAGGCACCACCCCTTGGAACATTTTCCAGTCATCTCA
E Q L V C W Q A G T T P W N I F P V I S
CTCTACCTAATGGGTGAGGTTACCAACCAGTCCTTCCGCATCACCATCCTTCCGCAGCAA
L Y L M G E V T N Q S F R I T I L P Q Q
TACCTGCGGCCAGTGAAGATGTGGCCACGTCCCAAGACGACTGTTACAAGTTTGCCATC

FIGURE 2 (2)

Y L R P V E D V A T S Q D D C Y K F A I
TCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAGGGCTTCTACGTTGTC
S Q S S T G T V M G A V I M E G F Y V V
TTTGATCGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGCCATGTGCACGATGAG
F D R A R K R I G F A V S A C H V H D E
TTCAGGACGGCAGCGGTGGAAGGCCCTTTTGTACCTTGGACATGGAAGACTGTGGCTAC
F R T A A V E G P F V T L D M E D C G Y
AACATTCCACAGACAGATGAGTCAACCCCTCATGACCATAGCCTATGTCATGGCTGCCATC
N I P Q T D E S T L M T I A Y V M A A I
TGCGCCCTCTTCATGCTGCCACTCTGCCTCATGGTGTGTGTCAGTGGCGCTGCCTCCGCTGC
C A L F M L P L C L M V C Q W R C L R C
CTGCGCCAGCAGCATGATGACTTTGCTGATGACATCTCCCTGCTGAAGTGAGGAGGCCCA
L R Q Q H D D F A D D I S L L K
TGGGCAGAAGATAGAGATTCCCTTGGACCACACCTCCGTGGTTCACTTTGGTCACAAGTA
GGAGACACAGATGGCACCTGTGGCCAGAGCACCTCAGGACCTTCCCCACCCACCAAATGC
CTCTGCCTTGATGGAGAAGGAAAAGGCTGGCAAGGTGGGTTCAGGGACTGTACCTGTAG
GAAACAGAAAAGAGAAGAAAGAAGCACTCTGCTGGCGGGAATACTCTTGGTCACCTCAA
TTTAAGTCGGGAAATCTGCTGCTTGAAACTTCAGCCCTGAACCTTTGTCCACCATTCCT
TTAAATTCCTCAACCCAAAGTATTCTTTCTTTCTTAGTTTCAGAAGTACTGGCATCACAC
GCAGGTTACCTTGGCGTGTGTCCCTGTGGTACCCTGGCAGAGAAGAGACCAAGCTTGT
CCCTGCTGGCCAAAGTCAGTAGGAGAGGATGCACAGTTTGCTATTTGCTTTAGAGACAGG
GACTGTATAAACAAGCCTAACATTGGTGCAAAGATTGCCTCTTGAAAAAAAAAAAAA

FIGURE 3 (1)

ATGGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGGGGAGTGCTGCCTGCCAC
M A Q A L P W L L L W M G A G V L P A H
GGCACCCAGCACGGCATCCGGCTGCCCTGCGCAGCGGCTGGGGGGCGCCCCCTGGGG
G T Q H G I R L P L R S G L G G A P L G
CTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGCCGGAGGGGAGCTTT
L R L P R E T D E E P E E P G R R G S F
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC
V E M V D N L R G K S G Q G Y Y V E M T
GTGGGCAGCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACCTTTGCA
V G S P P Q T L N I L V D T G S S N F A
GTGGGTGCTGCCCCCACCCTTCTGTCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V G A A P H P F L H R Y Y Q R Q L S S T
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAG
Y R D L R K G V Y V P Y T Q G K W E G E
CTGGGCACCGACCTGGTAAGCATCCCCATGGCCCCAACGTCACGTGCGTGCCAACATT
L G T D L V S I P H G P N V T V R A N I
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAAGTGGGAAGGCATCCTG
A A I T E S D K F F I N G S N W E G I L
GGGCTGGCCTATGCTGAGATTGCCAGGCCTGACGACTCCCTGGAGCCTTTCTTTGACTCT
G L A Y A E I A R P D D S L E P F F D S
CTGGTAAAGCAGACCCACGTTCCCAACCTCTTCTCCCTGCAGCTTTGTGGTGTGGCTTC
L V K Q T H V P N L F S L Q L C G A G F
CCCCTCAACCAGTCTGAAGTGCTGGCCTCTGTGCGGAGGGAGCATGATCATTGGAGGTATC
P L N Q S E V L A S V G G S M I I G G I
GACCACTCGCTGTACACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTAT
D H S L Y T G S L W Y T P I R R E W Y Y
GAGGTATCATTGTGCGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAG
E V I I V R V E I N G Q D L K M D C K E
TACAACTATGACAAGAGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAA
Y N Y D K S I V D S G T T N L R L P K K
GTGTTTGAAGCTGCAGTCAAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCTGAT
V F E A A V K S I K A A S S T E K F P D

FIGURE 3 (2)

GGTTTCTGGCTAGGAGAGCAGCTGGTGTGCTGGCAAGCAGGCACCACCCCTTGGAAACATT
G F W L G E Q L V C W Q A G T T P W N I

TTCCAGTCATCTCACTCTACCTAATGGGTGAGGTACCAACCAGTCCTTCCGCATCACC
F P V I S L Y L M G E V T N Q S F R I T

ATCCTTCCGCAGCAATACCTGCGGCCAGTGAAGATGTGGCCACGTCCAAGACGACTGT
I L P Q Q Y L R P V E D V A T S Q D D C

TACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAG
Y K F A I S Q S S T G T V M G A V I M E

GGCTTCTACGTTGTCTTTGATCGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGC
G F Y V V F D R A R K R I G F A V S A C

CATGTGCACGATGAGTTCAGGACGGCAGCGGTGAAGGCCCTTTTGTACCTTGGACATG
H V H D E F R T A A V E G P F V T L D M

GAAGACTGTGGCTACAACATTCCACAGACAGATGAGTCAACCCTCATGACCATAGCCTAT
E D C G Y N I P Q T D E S T L M T I A Y

GTCATGGCTGCCATCTGCGCCCTCTTCATGCTGCCACTCTGCCTCATGGTGTGTCAGTGG
V M A A I C A L F M L P L C L M V C Q W

CGCTGCCTCCGCTGCCTGCGCCAGCAGCATGATGACTTTGCTGATGACATCTCCCTGCTG
R C L R C L R Q Q H D D F A D D I S L L

AAGTGAGGAGGCCCATGGGCAGAAGATAGAGATTCCCCTGGACCACACCTCCGTGGTTCA
K

CTTTGGTCACAAGTAGGAGACACAGATGGCACCTGTGGCCAGAGCACCTCAGGACCCTCC
CCACCCACCAAATGCCTCTGCCTTGATGGAGAAGGAAAAGGCTGGCAAGGTGGGTTCAG
GGACTGTACCTGTAGGAAACAGAAAAGAGAAGAAAGAAGCACTCTGCTGGCGGGAATACT
CTTGGTACCTCAAATTTAAGTCGGGAAATTCTGCTGCTTGAAACTTCAGCCCTGAACCT
TTGTCCACCATTCCCTTAAATTCTCCAACCCAAAGTATTCTTCTTTTCTTAGTTTCAGAA
GTACTGGCATCACACGCAGGTTACCTTGGCGTGTGTCCTGTGGTACCCTGGCAGAGAAG
AGACCAAGCTTGTTPCCCTGCTGGCCAAAGTCAGTAGGAGAGGATGCACAGTTTGCTATT
TGCTTTAGAGACAGGGACTGTATAACAAGCCTAACATTGGTGCAAAGATTGCCTCTTGA
ATTA

FIGURE 4

ATGGCCCCAGCGCTGCTGCTGCTGCTATGGGTGGGCTCGGGAATGCTGCTGCCCCAG
M A P A L H W L L L W V G S G M L P A Q
GGAACCCATCTCGGCATCCGGCTGCCCTTCGCAGCGGCTGGCAGGGCCACCCCTGGGC
G T H L G I R L P L R S G L A G P P L G
CTGAGGCTGCCCCGGGAGACTGACGAGGAATCGGAGGAGCCTGGCCGGAGAGGCAGCTTT
L R L P R E T D E E S E E P G R R G S F
GTGGAGATGGTGGACAACCTGAGGGGAAAGTCCGGCCAGGGCTACTATGTGGAGATGACC
V E M V D N L R G K S G Q G Y Y V E M T
GTAGGCAGCCCCCACAGACGCTCAACATCCTGGTGGACACGGGCAGTAGTAACCTTGCA
V G S P P Q T L N I L V D T G S S N F A
GTGGGGCTGCCCCACACCCCTTCTGTCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V G A A P H P F L H R Y Y Q R Q L S S T
TATCGAGACCTCCGAAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAGGGGAA
Y R D L R K G V Y V P Y T Q G K W E G E
CTGGGCACCGACCTGGTGAGCATCCCTCATGGCCCCAACGTCACTGTGCGTGCCAAACATT
L G T D L V S I P H G P N V T V R A N I
GCTGCCATCACTGAATCGGACAAAGTTCTTCAATCAATGGTTCAACTGGGAGGGCATCCTA
A A I T E S D K F F I N G S N W E G I L
GGGCTGGCCTATCTGAGATTCCAGGCCCGACGACTCTTTGGAGCCCTTCTTTGACTCC
G L A Y A E I A R P D D S L E P F F D S
CTGGTGAAGCAGACCCACATTCCCAACATCTTTCCCTGCAGCTCTGTGGCGCTGGCTTC
L V K Q T H I P N I F S L Q L C G A G F
CCCCTCAACCAGACCGAGGCAGTGGCCTCGGTGGGAGGAGCATGATCATTGGTGTATC
P L N Q T E A L A S V G G S M I I G G I
GACCACTCGCTATACACGGGCGAGTCTCTGGTACACACCCATCCGGCGGGAGTGGTATTAT
D H S L Y T G S L W Y T P I R R E W Y Y
GAAGTGATCTTGTACCTGTGGAAATCAATGGTCAAGATCTCAAGATGGACTGCAAGGAG
E V I I V R V E I N G Q D L K M D C K E
TACAACACGACAAGAGCATTGTGGACAGTGGGACCACCAACCTTCGCTTGCCCCAAGAAA
Y N Y D K S I V D S G T T N L R L P K K
GTATTTGAAGCTGCGCTCAAGTCCATCAAGGCAGCCTCCTCGACGGAGAAGTTCCCGGAT
V F E A A V K S I K A A S S T E K F P D
GGCTTTTGGCTAGGGGACGAGCTGGTGTGTGGCAAGCAGGACGACCCCTTGGAACATT
G F W L G E Q L V C W Q A G T T P W N I
TTCCCACTATTTCATTTACCTCATGGGTGAAGTCACCAATCAGTCTTCCGCATCACC
F P V I S L Y L M G E V T N Q S F R I T
ATCCTTCTCAGCAATACCTACGGCCGGTGGAGGACGTGGCCACGTCCTCCCAAGACGACTGT
I L P Q Q Y L R P V E D V A T S Q D D C
TACAAGTTCGCTGTCTCACAGTCATCCACGGGCACTGTTATGGGAGCGGTCACTATGGAA
Y K F A V S Q S S T G T V M G A V I M E
GGTITCTATGTGCTTTCGATCGAGCCCGAAAGCGAATTGGCTTTGCTGTACGCGCTTGC
G F Y V V F D R A R K R I G F A V S A C
CATGTGACGATGAGTTCAGGACGGCGGCACTGGAAGGTCCGTTTGTTACGGCAGACATG
H V H D E F R T A A V E G P F V T A D M
GAAGACTGTGGCTACAAACATTCCCCAGACAGATGAGTCAACACTTATGACCATAGCCTAT
E D C G Y N I P Q T D E S T L M T I A Y
GTCATGGCGGCATCTGCGCCCTCTTCATGTTGCCACTCTGCCTCATGGTATGTCAGTGG
V M A A I C A L F M L P L C L M V C Q W
CGCTGCTGCTGCTGCTGCGCCACGACGATGACTTTGCTGATGACATCTCCCTGCTC
R C L R C L R H Q H D D F A D D I S L L
AAGTAAGGAGCTCCTGGGCAGATGATGGAGACGCCCTGGACCACATCTGGGTGGTTCC
K
CTTTGGTCAATGAGTTGGAGCTATGGATGGTACCTGTGGCCAGAGCACCTCAGGACCCCT
CACCAACCTGCCAATGCTTCTGGCGTGACAGAACAGAGAAATCAGGCAAGCTGGATTACA
GGGCTTGCACCTGTAGGACACAGGAGAGGGAAAGGAAGCAGCGTTCTGGTGGCAGGAATAT
CCTTAGGCACCAAACTTGAGTTGGAAATTTTGTGCTTGAAGCTTCAGCCCTGACCCCT
CTGCCACGATCCCTTAGAGTCTCCAACCTAAAGTATCTTTATGTCCTTCCAGAAGTAC
TGGCGTCATCTCAGGCTACCCGGCATGTGTCCTGTGGTACCCCTGGCAGAGAAAGGGCC
AATCTCATTCCCTGCTGGCCAAAGTCAGCAGAAGAGGTGAAGTTGCCAGTTGCTTTAG
TGATAGGACTGCAGACTCAAGCCCTACACTGGTACAAAGACTGCGCTTTGAGATAAACAA
GAA

FIGURE 5

```
1 MAQALPWLLLLWMGAGVLPAGHTQHGIRLPLRSGLGGAFLGLRLPRETDEE 50
  |||||
1 MAPALHWLLLLWVGSGMLPAQGTHLGIRLPLRSGLAGPPLGLRLPRETDEE 50
  |||||
51 PEEPGRRGSFVEMVDNLRGKSGQGYVEMTVGSPQTNLILVDTGSSNFA 100
  |||||
51 SEEPGRRGSFVEMVDNLRGKSGQGYVEMTVGSPQTNLILVDTGSSNFA 100
  |||||
101 VGAAPHFPLHRYYQRLSSTYRDLRKGYYVPYTOGKWEGLGTDLVSI PH 150
  |||||
101 VGAAPHFPLHRYYQRLSSTYRDLRKGYYVPYTOGKWEGLGTDLVSI PH 150
  |||||
151 GPNVTVRANIAAITESDKFFINGSNWEGILGLAYAEIARPDDSLEPFFDS 200
  |||||
151 GPNVTVRANIAAITESDKFFINGSNWEGILGLAYAEIARPDDSLEPFFDS 200
  |||||
201 LVKQTHVPNLFSLQCGAGFPLNQSEVLASVGGSMIIGGIDHSlyTGSLW 250
  |||||
201 LVKQTHIPNIFSLQCGAGFPLNQTEALASVGGSMIIGGIDHSlyTGSLW 250
  |||||
251 YTPIRREWYYEVIIVRVEINGQDLKMDCKEYNYDKSIVDSGTTNLR LPKK 300
  |||||
251 YTPIRREWYYEVIIVRVEINGQDLKMDCKEYNYDKSIVDSGTTNLR LPKK 300
  |||||
301 VFEEAAVKSIIKAASSTEKFPDGFWLGEQLVCWQAGTTPWNIFPVISLY LMG 350
  |||||
301 VFEEAAVKSIIKAASSTEKFPDGFWLGEQLVCWQAGTTPWNIFPVISLY LMG 350
  |||||
351 EVTNQSFRTILPQQYLRPVEDVATSQDDCYKFAISQSSTGTVMGAVIME 400
  |||||
351 EVTNQSFRTILPQQYLRPVEDVATSQDDCYKFAVSQSSTGTVMGAVIME 400
  |||||
401 GFYVVFDRAKRIGFAVSACHVHDEFRTAAVEGPFVTLDMEDCGYNIPQT 450
  |||||
401 GFYVVFDRAKRIGFAVSACHVHDEFRTAAVEGPFVTADMEDCGYNIPQT 450
  |||||
451 DESTLMTIAYVMAAICALFMLPLCLMVCQWRCLRLRQOHDDFADDISLL 500
  |||||
451 DESTLMTIAYVMAAICALFMLPLCLMVCQWRCLRLRHQOHDDFADDISLL 500
  |||||
501 K 501
  |
501 K 501
```

FIGURE 6 (1)

ATGGCTAGCATGACTGGTGGACAGCAAATGGGTGCGGGATCCACCCAGCAACGGCATCCGG
 M A S M T G G Q Q M G R G S T Q H G I R
 CTGCCCCTGCGCAGCGGCTGGGGGGCGCCCCCTGGGGCTGCGGCTGCCCCGGGAGACC
 L P L R S G L G G A P L G L R L P R E T
 GACGAAGAGCCCGAGGAGCCCGGCGGAGGGGAGCTTTGTGGAGATGGTGGACAACCTG
 D E E P E E P G R R G S F V E M V D N L
 AGGGGCAAGTCGGGCGAGGGCTACTACGTGGAGATGACCGTGGGCGAGCCCCCGCAGACG
 R G K S G Q G Y Y V E M T V G S P P Q T
 CTC AACATCCTGGTGGATACAGGCAGCAGTAACCTTTCAGTGGGTGCTGCCCCCACCCC
 L N I L V D T G S S N F A V G A A P H P
 TTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACATACCGGACCTCCGGAAGGGC
 F L H R Y Y Q R Q L S S T Y R D L R K G
 GTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAGCTGGGCGACCGACCTGGTAAGC
 V Y V P Y T Q G K W E G E L G T D L V S
 ATCCCCCATGGCCCCAACGTCACTGTGCGTCCCAACATTGCTGCCATCACTGAATCAGAC
 I P H G P N V T V R A N I A A I T E S D
 AAGTTCTTCATCAACGGCTCCAACTGGGAAGGCATCCTGGGGCTGGCCTATGCTGAGATT
 K F F I N G S N W E G I L G L A Y A E I
 GCCAGGCTGACGACTCCCTGGAGCCTTTCTTTGACTCTCTGGTAAAGCAGACCCACGTT
 A R P D D S L E P F F D S L V K Q T H V
 CCCAACCTCTTCTCCCTCAGCTTTGTGGTGTGGCTTCCCCCTCAACAGTCTGAAGTG
 P N L F S L Q L C G A G F P L N Q S E V
 CTGGCCTCTGTGCGAGGGAGCATGATCATTTGGAGGTATCGACCACTCGCTGTACACAGGC
 L A S V G G S M I I G G I D H S L Y T G
 AGTCTCTGGTATACACCCATCCGGCGGAGTGGTATTATGAGGTCATCATTTGTGCGGGTG
 S L W Y T P I R R E W Y Y E V I I V R V
 GAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAGTACAACATGACAAGAGCATT
 E I N G Q D L K M D C K E Y N Y D K S I
 CTGGACAGTGGCACCACCAACCTTCGTTTCCCCAAGAAAGTGTTTGAAGCTGCAGTCAAA
 V D S G T T N L R L P K K V F E A A V K
 TCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCTGATGGTTTCTGGCTAGGAGAGCAG
 S I K A A S S T E K F P D G F W L G E Q
 CTGGTGTGCTGGCAAGCAGGCACACCCCTTGGAAACATTTTCCAGTTCATCTCACTCTAC
 L V C W Q A G T T P W N I F P V I S L Y
 CTAATGGGTGAGCTTACCAACCAGTCCTTCCGCATCACCATCCTTCCGCAGCAATACCTG
 L M G E V T N Q S F R I T I L P Q Q Y L
 CGGCCAGTGAAGATGTGGCCACGTCCCAAGACGACTGTTACAAGTTTGCCATCTCACAG

FIGURE 6 (2)

R P V E D V A T S Q D D C Y K F A I S Q
TCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAGGGCTTCTACGTTGCTTTTGAT
S S T G T V M G A V I M E G F Y V V F D
CGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGCCATGTGCACGATGAGTTCAGG
R A R K R I G F A V S A C H V H D E F R
ACGGCAGCGGTGGAAGGCCCTTTTGTACCTTGGACATGGAAGACTGTGGCTACAACATT
T A A V E G P F V T L D M E D C G Y N I
CCACAGACAGATGATCATGA
P Q T D E S *

FIGURE 7 (1)

ATGGCTAGCATGACTGGTGGACAGCAAATGGGTGCGGATCGATGACTATCTTGACTCT
M A S M T G G Q Q M G R G S M T I S D S

CCGCGTGAACAGGACGGATCCACCCAGCAAGGCATCCGGCTGCCCTGGCAGCGGCCTG
P R E Q D G S T Q H G I R L P L R S G L

GGGGGCGCCCCCTGGGGCTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCCGAGGAGCCC
G G A P L G L R L P R E T D E E P E E P

GGCCGAGGGGCGAGCTTTGTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGCGAGGGC
G R R G S F V E M V D N L R G K S G Q G

TACTACGTGGAGATGACCGTGGGCGAGCCCCCGCAGACGCTCAACATCCTGGTGGATACA
Y Y V E M T V G S P P Q T L N I L V D T

GGCAGCAGTAACCTTTGCAGTGGGTGCTGCCCCCACCCTTCCTGCATCGCTACTACCAG
G S S N F A V G A A P H P F L H R Y Y Q

AGGCAGCTGTCCAGCACATACCGGGACCTCCGGAAGGGCGTGTATGTGCCCTACACCCAG
R Q L S S T Y R D L R K G V Y V P Y T Q

GGCAAGTGGGAAGGGGAGCTGGGCACCGACCTGGAAGCATCCCCCATGGCCCCAACGTC
G K W E G E L G T D L V S I P H G P N V

ACTGTGCGTGCCAACATTGCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCC
T V R A N I A A I T E S D K F F I N G S

AACTGGGAAGGCATCCTGGGGCTGGCCTATGCTGAGATTGCCAGGCCTGACGACTCCCTG
N W E G I L G L A Y A E I A R P D D S L

GAGCCTTTCTTTGACTCTCTGGTAAAGCAGACCCACGTTCCCAACCTCTTCTCCCTGCAG
E P F F D S L V K Q T H V P N L F S L Q

CTTTGTGGTGCTGGCTTCCCCCTCAACCACTCTGAAGTGCTGGCCTCTGTGGAGGGAGC
L C G A G F P L N Q S E V L A S V G G S

ATGATCATGGAGGTATCGACCACTCGCTGTACACAGGCAGTCTCTGGTATACACCCATC
M I I G G I D H S L Y T G S L W Y T P I

CGGCGGAGTGGTATTATGAGGTCATCATTGTGCGGGTGGAGATCAATGGACAGGATCTG
R R E W Y Y E V I I V R V E I N G Q D L

AAAAATGGACTGCAAGGAGTACAACATGACAAGAGCATTGTGGACAGTGGCACCACCAAC
K M D C K E Y N Y D K S I V D S G T T N

CTTCGTTTCCCCAAGAAAGTGTGGAAGCTGCAGTCAAATCCATCAAGGCAGCCTCCTCC
L R L P K K V F E A A V K S I K A A S S

ACGGAGAAGTTCCCTGATGGTTTCTGGCTAGGAGAGCAGCTGGTGTGCTGGCAAGCAGGC
T E K F P D G F W L G E Q L V C W Q A G

ACCACCCCTTGGAAACATTTTCCAGTCATCTCACTTACCTAATGGGTGAGGTTACCAAC
T T P W N I F P V I S L Y L M G E V T N

FIGURE 7 (2)

CAGTCCTTCCGCATCACCATCCTTCCGCAGCAATACCTGCGGCCAGTGGGAAGATGTGGCC
Q S F R I T I L P Q Q Y L R P V E D V A
ACGTCCCAAGACGACTGTTACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATG
T S Q D D C Y K F A I S Q S S T G T V M
GGAGCTGTTATCATGGAGGGCTTCTACGTTGTCTTTGATCGGGCCCGAAAACGAATTGGC
G A V I M E G F Y V V F D R A R K R I G
TTTGCTGTCAGCGCTTGCCATGTGCACGATGAGTTCAGGACGGCAGCGGTGGAAGGCCCT
F A V S A C H V H D E F R T A A V E G P
TTTGTCACCTTGACATGGAAGACTGTGGCTACAACATTCCACAGACAGATGASTCATGA
F V T L D M E D C G Y N I P Q T D E S *

FIGURE 8 (1)

ATGACTCAGCATGGTATTGCTCTGCCACTGCCGTAGCGCTCTGGGTGGTGCTCCACTGGGT
M T Q H G I R L P L R S G L G G A P L G -
CTGCGTCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGCCGGAGGGGCAGCTTT
L R L P R E T D E E P E E P G R R G S F -
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC
V E M V D N L R G K S G Q G Y Y V E M T -
GTGGGCAGCCCCCGCAGACGCTCAACATCCTGTGGATACAGGCAGCAGTAACTTTGCA
V G S P P Q T L N I L V D T G S S N F A -
GTGGGTGCTGCCCCCACCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V G A A P H P F L H R Y Y Q R Q L S S T -
TACCGGACCTCCGAAGGGCGTGTATGTCCCTACACCAGGGCAAGTGGGAAGGGGAG
Y R D L R K G V Y V P Y T Q G K W E G E -
CTGGGCAGCGACCTGGTAAGCATCCCCATGGCCCCAACGTCACTGTGCGTGCCCAACATT
L G T D L V S I P H G P N V T V R A N I -
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAACTGGGAAGGCATCCTG
A A I T E S D K F F I N G S N W E G I L -
GGGCTGGCCTATGCTGAGATTGCCAGGCCTGACGACTCCCTGGAGCCTTTCTTTGACTCT
G L A Y A E I A R P D D S L E P F F D S -
CTGGTAAAGCAGACCCACGTTCCCAACCTCTTCTCCCTGCAGCTTTGTGGTGCTGGCTTC
L V K Q T H V P N L F S L Q L C G A G F -
CCCCCAACCACTGTGAAGTCTGGCCTCTGTGCGGAGGAGCATGATCATGGAGGTATC
P L N Q S E V L A S V G G S M I I G G I -
GACCACTCGCTGTACACAGGCAGTCTCTGGTATACACCCATCCGGCGGAGTGGTATTAT
D H S L Y T G S L W Y T P I R R E W Y Y -
GAGGTCAATCATGTGCGGGTGGAGATCAATGGACAGGATCTGAAATGGACTGCAAGGAG
E V I I V R V E I N G Q D L K M D C K E -
TACAACTATGACAAGAGCATGTGGACAGTGGCACCACCAACCTTCGTTTGGCCCAAGAAA
Y N Y D K S I V D S G T T N L R L P K K -
GTGTTGAAGCTGCAGTCAAATCCATCAAGGCAGCCTCCTCCAGGAGAAGTTCCTGAT
V F E A A V K S I K A A S S T E K F P D -
GGTTTCTGGCTAGGAGAGCAGCTGGTGTGCTGGCAAGCAGGCACCCCTTGGAAACATT
G F W L G E Q L V C W Q A G T T P W N I -
TTCCCACTCATCTCACTCTACCTAATGGGTGAGGTTACCAACCACTCCTTTGCGCATCACC
F P V I S L Y L M G E V T N Q S F R I T -
ATCCTTCCGCAGCAATACCTGCGGCCAGTGGAAGATGTGGCCACGTCCCAAGACGACTGT
I L P Q Q Y L R P V E D V A T S Q D D C -

FIGURE 8 (2)

TACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAG
Y K F A I S Q S S T G T V M G A V I M E -
GGCTTCTACGTTGTCTTTGATCGGGCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGC
G F Y V V F D R A R K R I G F A V S A C -
CATTAG
H *

FIGURE 9

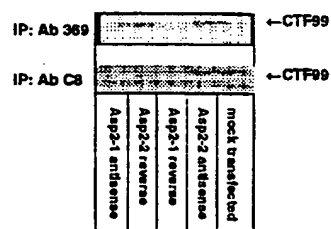


FIGURE 10

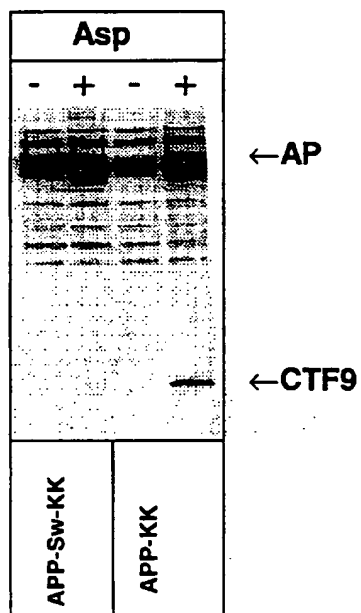


FIGURE 11

MAQALPWLLLWMGAGVLPAGHTQHGIRLPLRSGLGGAFLGLRLPRETDEE
PEEPGRRGSFVEMVDNLRGKSGQGYVEMTVGSPPTLNILVDTGSSNFA
VGAAPHPLHRYRQLSSTYRDLRKGYYVPYTOGKWEGLGTDLVSI PH
GPNVTVRANIAAITESDKFFINGSNWEGILGLAYAEIARPDDSLEPPFDS
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FIGURE 12

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SEQUENCE LISTING

<110> Gurney, Mark E.

Bienkowski, Michael J.

Heinrikson, Robert L.

Parodi, Luis A.

Yan, Riqiang

Pharmacia & Upjohn Company

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Pro Gly Thr Pro Ala Glu Arg His Ala Asp Gly Leu Ala Leu Ala Leu

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Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val

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Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser

305 310 315 320

Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile

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Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln

340 345 350

Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val

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<211> 501

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<213> Mus musculus

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35 40 45

Glu Glu Ser Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val

50 55 60

Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr

65 70 75 80

Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser

85 90 95

Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr

100 105 110

Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val

115 120 125

Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp

130 135 140

Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile

145 150 155 160

Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp
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 Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp Lys Ser Ile Val
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 Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys Val Phe Glu Ala
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 Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu Lys Phe Pro Asp
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 Gly Phe Trp Leu Gly Glu Gln Leu Val Cys Trp Gln Ala Gly Thr Thr

325 330 335
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Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu Pro Gln Gln Tyr Leu Arg
355 360 365
Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys Phe Ala
370 375 380
Val Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val Ile Met Glu
385 390 395 400
Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg Ile Gly Phe Ala
405 410 415
Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr Ala Ala Val Glu
420 425 430
Gly Pro Phe Val Thr Ala Asp Met Glu Asp Cys Gly Tyr Asn Ile Pro
435 440 445
Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val Met Ala Ala
450 455 460
Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val Cys Gln Trp
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485 490 495

Ile Ser Leu Leu Lys

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<211> 2088

<212> DNA

<213> Homo sapiens

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<210> 10

<211> 695

<212> PRT

<213> Homo sapiens

<400> 10

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20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln

35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
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 Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65 70 75 80
 Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
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 Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
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 Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
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 Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
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 Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
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 165 170 175
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
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 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys

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Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
      275              280              285
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Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
      340              345              350
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Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
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Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
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Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
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Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
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 Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
 595 600 605
 -
 His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
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 -
 Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
 625 630 635 640
 -
 Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
 645 650 655
 -
 His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
 660 665 670
 -
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20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln

35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp

50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu

65 70 75 80


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Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
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-
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
-
-           100           105           110
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-
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
-
-           115           120           125
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-
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
-
-           130           135           140
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Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
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145           150           155           160
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-
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
-
-           165           170           175
-
-
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
-
-           180           185           190
-
-
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
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-           195           200           205
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-
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
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Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
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225           230           235           240
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Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg

275 280 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu

290 295 300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys

305 310 315 320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg

325 330 335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp

340 345 350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu

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Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala

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Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn

385 390 395 400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe

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- Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
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- Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
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- Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
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- Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
-           500           505           510
-
- Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
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- Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
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- Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
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- Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
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580

585

590

Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val

595

600

605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys

610

615

620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val

625

630

635

640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile

645

650

655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg

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665

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His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys

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<213> Homo sapiens

<400> 14

Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg

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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro

20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln

35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp

50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu

65 70 75 80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn

85 90 95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val

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      100              105              110
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Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
      115              120              125
-
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
      130              135              140
-
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
      145              150              155              160
-
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
      165              170              175
-
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
      180              185              190
-
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
      195              200              205
-
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
      210              215              220
-
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
      225              230              235              240
-
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
      245              250              255
-
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
      260              265              270
-

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Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
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275 280 285
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Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
-
290 295 300
-
Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
-
305 310 315 320
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Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
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325 330 335
-
Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
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340 345 350
-
Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
-
355 360 365
-
Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
-
370 375 380
-
Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
-
385 390 395 400
-
Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
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405 410 415
-
Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
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420 425 430
-

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Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala

435 440 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu

450 455 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala

465 470 475 480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn

485 490 495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser

500 505 510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr

515 520 525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln

530 535 540

Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn

545 550 555 560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr

565 570 575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser

580 585 590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val

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      595              600              605
-
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
-      610              615              620
-
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
-      625              630              635              640
-
Ile Phe Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
-      645              650              655
-
His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
-      660              665              670
-
His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
-      675              680              685
-
Phe Phe Glu Gln Met Gln Asn
-      690              695
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<210> 15

<211> 2094

<212> DNA

<213> Homo sapiens

<400> 15

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ctgaacatgc acatgaatgt ccagaatggg aagtgggatt cagatccatc agggaccaa 180
acctgcattg ataccaagga aggcacccctg cagtattgcc aagaagtcta ccctgaactg 240

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 aagagtacca acttgcatga ctacggcatg ttgctgccct gcggaattga caagtccga 540
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<210> 16

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<213> Homo sapiens

<400> 16

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20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln

35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp

50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu

65 70 75 80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn

85 90 95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val

100 105 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu

115 120 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys

130 135 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu

145 150 155 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile

165 170 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu

180 185 190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val

195 200 205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys

210 215 220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu

225 230 235 240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu

245 250 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile

260 265 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg

275 280 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu

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-
Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305              310              315              320
-
-
Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
      325              330              335
-
-
Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
      340              345              350
-
-
Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
      355              360              365
-
-
Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
      370              375              380
-
-
Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385              390              395              400
-
-
Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
      405              410              415
-
-
Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
      420              425              430
-
-
Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
      435              440              445
-
-
Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
      450              455              460
-

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-
Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala

465 470 475 480

-
Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn

485 490 495

-
Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser

500 505 510

-
Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr

515 520 525

-
Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln

530 535 540

-
Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn

545 550 555 560

-
Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr

565 570 575

-
Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser

580 585 590

-
Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val

595 600 605

-
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys

610 615 620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val

625 630 635 640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile

645 650 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg

660 665 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys

675 680 685

Phe Phe Glu Gln Met Gln Asn Lys Lys

690 695

<210> 17

<211> 2094

<212> DNA

<213> Homo sapiens

<400> 17

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 aagagtacca acttgcata ctacggcatg ttgctgccct gcggaattga caagtccga 540

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 cctacgaag aagccacaga gagaaccacc agcattgccca ccaccaccac caccaccaca 840
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<212> PRT

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Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg

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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro

20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln

35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp

50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu

65 70 75 80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn

85 90 95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val

100 105 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu

115 120 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys

130 135 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu

145 150 155 160

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-
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
-
-           165               170               175
-
-
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
-
-           180               185               190
-
-
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
-
-           195               200               205
-
-
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
-
-           210               215               220
-
-
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
-
-           225               230               235               240
-
-
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
-
-           245               250               255
-
-
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
-
-           260               265               270
-
-
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
-
-           275               280               285
-
-
Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
-
-           290               295               300
-
-
Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
-
-           305               310               315               320
-
-

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Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg

325 330 335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp

340 345 350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu

355 360 365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala

370 375 380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn

385 390 395 400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe

405 410 415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His

420 425 430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala

435 440 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu

450 455 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala

465 470 475 480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn

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Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
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-
Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
-           515           520           525
-
Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
-           530           535           540
-
Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545           550           555           560
-
Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
-           565           570           575
-
Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
-           580           585           590
-
Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
-           595           600           605
-
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
-           610           615           620
-
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625           630           635           640
-
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
-           645           650           655
-

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His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg

660

665

670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys

675

680

685

Phe Phe Glu Gln Met Gln Asn Lys Lys

690

695

<210> 19

<211> 2094

<212> DNA

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 gtggagggtg acgccgtgt caccacagag gagcgccacc tgtccaagat gcagcagaac 2040
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<210> 20

<211> 697

<212> PRT

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<400> 20

Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg

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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
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 Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
 35 40 45
 Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
 50 55 60
 Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65 70 75 80
 Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
 85 90 95
 Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
 100 105 110
 Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
 115 120 125
 Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
 130 135 140
 Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
 145 150 155 160
 Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
 165 170 175
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu


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      180              185              190
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-
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
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-
-
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
      210              215              220
-
-
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
      225              230              235              240
-
-
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
      245              250              255
-
-
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
      260              265              270
-
-
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
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-
Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
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Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
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Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
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Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
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Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu

355 360 365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala

370 375 380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn

385 390 395 400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe

405 410 415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His

420 425 430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala

435 440 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu

450 455 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala

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Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn

485 490 495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser

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Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
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 Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
 545 550 555 560
 Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
 565 570 575
 Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
 580 585 590
 Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
 595 600 605
 His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
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 Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
 625 630 635 640
 Ile Phe Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
 645 650 655
 His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
 660 665 670
 His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys

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        675                680                685

Phe Phe Glu Gln Met Gln Asn Lys Lys

        690                695

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<212> DNA
<213> Homo sapiens

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gacgaagagc ccgaggagcc cggccggagg ggcagctttg tggagatggt ggacaacctg 180
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<211> 446

<212> PRT

<213> Homo sapiens

<400> 22

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His Gly Ile Arg Leu Pro Leu Arg Ser Gly Leu Gly Gly Ala Pro Leu

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Gly Leu Arg Leu Pro Arg Glu Thr Asp Glu Glu Pro Glu Glu Pro Gly

35 40 45

Arg Arg Gly Ser Phe Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser

50 55 60

Gly Gln Gly Tyr Tyr Val Glu Met Thr Val Gly Ser Pro Pro Gln Thr

65 70 75 80

Leu Asn Ile Leu Val Asp Thr Gly Ser Ser Asn Phe Ala Val Gly Ala

85 90 95

Ala Pro His Pro Phe Leu His Arg Tyr Tyr Gln Arg Gln Leu Ser Ser

100 105 110

Thr Tyr Arg Asp Leu Arg Lys Gly Val Tyr Val Pro Tyr Thr Gln Gly

115 120 125

Lys Trp Glu Gly Glu Leu Gly Thr Asp Leu Val Ser Ile Pro His Gly

130 135 140

Pro Asn Val Thr Val Arg Ala Asn Ile Ala Ala Ile Thr Glu Ser Asp

145 150 155 160

Lys Phe Phe Ile Asn Gly Ser Asn Trp Glu Gly Ile Leu Gly Leu Ala

165 170 175

Tyr Ala Glu Ile Ala Arg Pro Asp Asp Ser Leu Glu Pro Phe Phe Asp

180 185 190

Ser Leu Val Lys Gln Thr His Val Pro Asn Leu Phe Ser Leu His Leu

195 200 205

Cys Gly Ala Gly Phe Pro Leu Asn Gln Ser Glu Val Leu Ala Ser Val

210 215 220

Gly Gly Ser Met Ile Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly

225 230 235 240

Ser Leu Trp Tyr Thr Pro Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile

245 250 255

Ile Val Arg Val Glu Ile Asn Gly Gln Asp Leu Lys Met Asp Cys Lys

260 265 270

Glu Tyr Asn Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu

275 280 285

Arg Leu Pro Lys Lys Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala

290 295 300

Ala Ser Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln

305 310 315 320

Leu Val Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val

325 330 335

Ile Ser Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile

340 345 350

Thr Ile Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr

355 360 365

Ser Gln Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly

370 375 380

Thr Val Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp

385 390 395 400

Arg Ala Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His

405 410 415

Asp Glu Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp

420 425 430

Met Glu Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser

435

440

445

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<211> 1380

<212> DNA

<213> Homo sapiens

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<210> 24

<211> 459

<212> PRT

<213> Homo sapiens

<400> 24

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Ile Ser Asp Ser Pro Arg Glu Gln Asp Gly Ser Thr Gln His Gly Ile

20 25 30

Arg Leu Pro Leu Arg Ser Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg

35 40 45

Leu Pro Arg Glu Thr Asp Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly

50 55 60

Ser Phe Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly

65 70 75 80

Tyr Tyr Val Glu Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile

85 90 95

Leu Val Asp Thr Gly Ser Ser Asn Phe Ala Val Gly Ala Ala Pro His

100 105 110

Pro Phe Leu His Arg Tyr Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg

115 120 125

Asp Leu Arg Lys Gly Val Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu

130 135 140

Gly Glu Leu Gly Thr Asp Leu Val Ser Ile Pro His Gly Pro Asn Val

145 150 155 160

Thr Val Arg Ala Asn Ile Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe

165 170 175

Ile Asn Gly Ser Asn Trp Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu

180 185 190

Ile Ala Arg Pro Asp Asp Ser Leu Glu Pro Phe Phe Asp Ser Leu Val

195 200 205

Lys Gln Thr His Val Pro Asn Leu Phe Ser Leu His Leu Cys Gly Ala

210 215 220

Gly Phe Pro Leu Asn Gln Ser Glu Val Leu Ala Ser Val Gly Gly Ser

225 230 235 240

Met Ile Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu Trp

245 250 255

Tyr Thr Pro Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val Arg

260 265 270

Val Glu Ile Asn Gly Gln Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn

275 280 285

Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro

290 295 300

Lys Lys Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser Ser

305 310 315 320

Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val Cys

325 330 335

Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser Leu

340 345 350

Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu

355 360 365

Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln Asp

370 375 380

Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val Met

385 390 395 400

Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala Arg

405 410 415

Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu Phe

420 425 430

Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu Asp

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Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser

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<210> 25

<211> 1302

<212> DNA

<213> Homo sapiens

<400> 25

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1302

<210> 26

<211> 433

<212> PRT

<213> Homo sapiens

<400> 26

Met Thr Gln His Gly Ile Arg Leu Pro Leu Arg Ser Gly Leu Gly Gly

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Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu Thr Asp Glu Glu Pro Glu

20 25 30

Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val Asp Asn Leu Arg

35 40 45

Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr Val Gly Ser Pro

50 55 60

Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser Ser Asn Phe Ala

65 70 75 80

Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr Tyr Gln Arg Gln

85 90 95

Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val Tyr Val Pro Tyr

100 105 110

Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp Leu Val Ser Ile

115 120 125

Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile Ala Ala Ile Thr

130 135 140

Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp Glu Gly Ile Leu

145 150 155 160

Gly Leu Ala Tyr Ala Glu Ile Ala Arg Pro Asp Asp Ser Leu Glu Pro

165 170 175

Phe Phe Asp Ser Leu Val Lys Gln Thr His Val Pro Asn Leu Phe Ser

180 185 190

Leu His Leu Cys Gly Ala Gly Phe Pro Leu Asn Gln Ser Glu Val Leu

195 200 205

Ala Ser Val Gly Gly Ser Met Ile Ile Gly Gly Ile Asp His Ser Leu

210 215 220

Tyr Thr Gly Ser Leu Trp Tyr Thr Pro Ile Arg Arg Glu Trp Tyr Tyr

225 230 235 240

Glu Val Ile Ile Val Arg Val Glu Ile Asn Gly Gln Asp Leu Lys Met

245 250 255

Asp Cys Lys Glu Tyr Asn Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr

260 265 270

Thr Asn Leu Arg Leu Pro Lys Lys Val Phe Glu Ala Ala Val Lys Ser

275 280 285

Ile Lys Ala Ala Ser Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu

290 295 300
-
Gly Glu Gln Leu Val Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile
305 310 315 320
-
Phe Pro Val Ile Ser Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser
325 330 335
-
Phe Arg Ile Thr Ile Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp
340 345 350
-
Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser
355 360 365
-
Ser Thr Gly Thr Val Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val
370 375 380
-
Val Phe Asp Arg Ala Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys
385 390 395 400
-
His Val His Asp Glu Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val
405 410 415
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Thr Leu Asp Met Glu Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu
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<210> 27
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<211> 1278

<212> DNA

<213> Homo sapiens

<400> 27

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1278

<210> 28

<211> 425

<212> PRT

<213> Homo sapiens

<400> 28

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20 25 30

Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr

35 40 45

Val Glu Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val

50 55 60

Asp Thr Gly Ser Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe

65 70 75 80

Leu His Arg Tyr Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu

85 90 95

Arg Lys Gly Val Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu

100 105 110

Leu Gly Thr Asp Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val

115 120 125

Arg Ala Asn Ile Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn

130 135 140

Gly Ser Asn Trp Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu Ile Ala

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145          150          155          160
-
-
Arg Pro Asp Asp Ser Leu Glu Pro Phe Phe Asp Ser Leu Val Lys Gln
-          165          170          175
-
-
Thr His Val Pro Asn Leu Phe Ser Leu His Leu Cys Gly Ala Gly Phe
-          180          185          190
-
-
Pro Leu Asn Gln Ser Glu Val Leu Ala Ser Val Gly Gly Ser Met Ile
-          195          200          205
-
-
Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu Trp Tyr Thr
-          210          215          220
-
-
Pro Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val Arg Val Glu
225          230          235          240
-
-
Ile Asn Gly Glu Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp
-          245          250          255
-
-
Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys
-          260          265          270
-
-
Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu
-          275          280          285
-
-
Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val Cys Trp Gln
-          290          295          300
-
-
Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser Leu Tyr Leu
305          310          315          320
-

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-
Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys

- 340 345 350

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Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala

- 355 360 365

-
Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg

- 370 375 380

-
Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr

- 385 390 395 400

-
Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu Asp Cys Gly

- 405 410 415

-
Tyr Asn Ile Pro Gln Thr Asp Glu Ser

- 420 425

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<210> 29

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<211> 1362

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<212> DNA

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<213> Homo sapiens

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<400> 29

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 Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr
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Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu Lys Phe Pro Asp
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355 360 365

Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys Phe Ala

370 375 380

Ile Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val Ile Met Glu

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<211> 77

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<211> 51

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<213> Artificial Sequence

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Caspase 8

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<210> 43

<211> 32

<212> DNA

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<210> 44

<211> 36

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<400> 44

gaaagcttcc atgactcacc tgtctgtgga atgttg 36

<210> 45

<211> 24

<212> DNA

<213> Artificial Sequence

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- motif
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csnayrsnay rh0dtgactg accactcgac caggttcact snayrtcsn asnanrmdt 180
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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Introduce KK

motif

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nayrsnayrh 0htcgaatta aattccagca cactggctac ttcttgttct gcatctcaa 240
gaacgaasna yrttcnash anmadtcsn ayrtcnamcr stwrd0cgks kdharmaca 300
hncgaattaa attccagcac actggctact tcttgttctg catctcaaag aacttdgdan 360
0b0cda00a0 ca0rtntryh kktabwrddc mntsmmaryn rmatndcmnt smmarynrma 420
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<211> 380

<212> DNA

<213> Artificial Sequence

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motif

<400> 49

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snasnarnma dtcsnayrtc namcrstwr 0cmdhharma cahncgaatt aaattccagc 240
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acactggcta ttgdgan0b0 cda00a0ca0 rtntrymknt abwrddcmnt smmarynrma 300
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dcmntmswrđ dcwrddcmnt 380



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 15/57, C07K 14/47, C07K 16/18, C07K 19/00, C12N 1/21, C12N 5/10, C12N 9/64, C12N 15/12, C12N 15/62, C12N 15/85, C12Q 1/37, G01N 33/68	A3	(11) International Publication Number: WO 00/17369 (43) International Publication Date: 30 March 2000 (30.03.2000)
(21) International Application Number: PCT/US99/20881 (22) International Filing Date: 23 September 1999 (23.09.1999) (30) Priority Data: 60/101,594 24 September 1998 (24.09.1998) US (60) Parent Application or Grant PHARMACIA & UPJOHN COMPANY [/]; O. GURNEY, Mark, E. [/]; O. BIENKOWSKI, Michael, Jerome [/]; O. HEINRIKSON, Robert, Leroy [/]; O. PARODI, Luis, A. [/]; O. YAN, Riqiang [/]; O. GURNEY, Mark, E. [/]; O. BIENKOWSKI, Michael, Jerome [/]; O. HEINRIKSON, Robert, Leroy [/]; O. PARODI, Luis, A. [/]; O. YAN, Riqiang [/]; O. WOOTTON, Thomas, A. ; O.		Published
(54) Title: ALZHEIMER'S DISEASE SECRETASE (54) Titre: SECRETASE DE LA MALADIE D'ALZHEIMER (57) Abstract <p>The present invention provides the enzyme and enzymatic procedures for cleaving the 'beta' secretase cleavage site of the APP protein and associated nucleic acids, peptides, vectors, cells and cell isolates and assays.</p> (57) Abrégé <p>La présente invention porte sur l'enzyme et les procédures enzymatiques de clivage du site de clivage de la 'beta' sécrétase de la protéine APP et des acides nucléiques, des peptides, des vecteurs, des cellules et des isolats cellulaires associés, et sur des dosages.</p>		

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(51) International Patent Classification ⁷ : C12N 15/57, 15/62, 15/85, 5/10, 9/64, C07K 19/00, 14/47, C12N 15/12, C07K 16/18, C12Q 1/37, G01N 33/68, C12N 1/21	A3	(11) International Publication Number: WO 00/17369 (43) International Publication Date: 30 March 2000 (30.03.00)
(21) International Application Number: PCT/US99/20881 (22) International Filing Date: 23 September 1999 (23.09.99) (30) Priority Data: 60/101,594 24 September 1998 (24.09.98) US (71) Applicant (for all designated States except US): PHARMACIA & UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GURNEY, Mark, E. [US/US]; 910 Rosewood Avenue, S.E., Grand Rapids, MI 49506 (US). BIENKOWSKI, Michael, Jerome [US/US]; 3431 Hollow Wood, Portage, MI 49024 (US). HEINRIK- SON, Robert, Leroy [US/US]; 81 South Lake Doster Drive, Plainwell, MI 49080 (US). PARODI, Luis, A. [US/SE]; Grevgafan 24, S-115 43 Stockholm (SE). YAN, Riqiang [US/US]; 5026 Queen Victoria Street, Kalamazoo, MI 49009 (US).	(74) Agent: WOOTTON, Thomas, A.; Pharmacia & Upjohn Com- pany, Intellectual Property Legal Services, 301 Henrietta Street, Kalamazoo, MI 49001 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 23 November 2000 (23.11.00)	
(54) Title: ALZHEIMER'S DISEASE SECRETASE (57) Abstract The present invention provides the enzyme and enzymatic procedures for cleaving the β secretase cleavage site of the APP protein and associated nucleic acids, peptides, vectors, cells and cell isolates and assays.		

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DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 99/20881

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/57	C12N15/62	C12N15/85	C12N5/10	C12N9/64
	C07K19/00	C07K14/47	C12N15/12	C07K16/18	C12Q1/37
	G01N33/68	C12N1/21			
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
IPC 7 C12N C07K C12Q G01N					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
EPO-Internal, STRAND, WPI Data, BIOSIS, CHEM ABS Data, MEDLINE, EMBL					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category *	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	<p>EP 0 848 062 A (SMITHKLINE-BEECHAM CORPORATION) 17 June 1998 (1998-06-17) cited in the application</p> <p>page 2, line 10 -page 3, line 40 page 4, line 20 - line 33 page 5, line 8 - line 20 page 8, line 1 -page 9, line 25; tables 1,2</p> <p style="text-align: center;">-/-</p>				<p>1-3, 5-21,24, 25, 28-31, 34, 37-47, 49-64, 66-69, 72-75, 77, 80-91, 95-97, 114-129, 140,141</p>
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.					
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Date of the actual completion of the international search			Date of mailing of the international search report		
26 July 2000			02.08.00		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patendean 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 851 epo nl Fax: (+31-70) 340-3018			Authorized officer Montero Lopez, B		

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INTERNATIONAL SEARCH REPORT

Internat. Appl. No.
PCT/US 99/20881

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>page 10, line 28 - line 44 page 11, line 10 -page 12, line 8</p> <p>EP 0 855 444 A (SMITHKLINE-BEECHAM P.L.C.) 29 July 1998 (1998-07-29) cited in the application</p>	<p>1-3, 5-21, 24, 25, 28-31, 34, 37-47, 49-64, 66-69, 72-75, 77, 80-91, 95-97, 114-129, 140, 141</p>
	<p>page 2, line 8 -page 3, line 44 page 5, line 3 - line 15 page 5, line 49 -page 6, line 3; tables 1, 2 page 7, line 34 - line 50 page 10, line 20 -page 11, line 1 page 12, line 1 - line 19 page 12, line 45 -page 13, line 44</p> <p>WO 96 40885 A (ATHENA NEUROSCIENCES) 19 December 1996 (1996-12-19)</p> <p>page 3, line 1 -page 5, line 26 page 8, line 1 - line 34 page 14, line 19 -page 17, line 22 page 23, line 31 -page 25, line 20 page 28, line 7 -page 48, line 13</p> <p style="text-align: center;">-- -/--</p>	<p>1-4, 6, 7, 9, 10, 12-21, 24, 25, 28-31, 34, 37-47, 49, 50, 52, 53, 55-63, 67, 68, 72-75, 77, 80-90, 108-129, 136-139, 141</p>

INTERNATIONAL SEARCH REPORT

Intern. Appl. No.
PCT/US 99/20881

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 98 26059 A (ATHENA NEUROSCIENCES, INC.) 18 June 1998 (1998-06-18)</p> <p>page 2, line 35 -page 4, line 3 page 5, line 9 -page 11, line 5 page 11, line 10 -page 22, line 3</p>	<p>1-4,6,7, 9,10, 12-21, 24,25, 28-31, 34, 37-47, 49,50, 52,53, 55-63, 67,68, 72-75, 77, 80-90, 108-129, 136-139, 141</p>
P,X	<p>WO 99 34004 A (CHIRON CORPORATION) 8 July 1999 (1999-07-08)</p> <p>page 7, line 19 -page 8, line 9 page 11, line 22 -page 14, line 24 page 16, line 26 -page 21, line 1 page 21, line 20 -page 23, line 13; figure 2; examples 2,3</p> <p>---</p> <p>-/-</p>	<p>1-4,6,7, 9-20,24, 28-31, 34, 37-47, 49,50, 52-63, 67,68, 72-75, 77, 80-92, 95-98, 101-103, 106,107, 114-117, 120,141</p>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/20881

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 46281 A (GENENTECH, INC.) 16 September 1999 (1999-09-16)	1-4, 6, 7, 9-12, 18-20, 24, 28-31, 34, 37, 38, 40-47, 49, 50, 52-54, 61-63, 67, 68, 72-75, 77, 80, 81, 84-92, 95-98, 101-103, 106, 107, 114-118, 120-128, 140, 141
	page 15, line 10 - line 23 page 65, line 5 - line 25 page 130, line 30 - line 35 page 149, line 3 - page 155, line 6 page 160, line 20 - line 22 page 173, line 35 - page 175, line 23; figures 72, 73; examples 32, 99-107	
A	US 5 795 963 A (MICHAEL JOHN MULLAN) 18 August 1998 (1998-08-18) column 3, line 58 - column 6, line 21	130-135, 141
T	YAN RUIJIANG ET AL.: "Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity" NATURE, vol. 402, 2 December 1999 (1999-12-02), pages 533-537, XP002136300 LONDON GB	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/28881

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 218

Continuation of Box 1.2

Claims Nos.: claims 32, 33, 35, 36, 70, 71, 76, 78 and 79 and partially claims 1, 18, 28, 44, 61, 72 and 141

Present claims 1, 18, 28, 44, 61, 72 and 141 relate to an extremely large number of possible products. In fact, the claims encompass so many possible compounds that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.

Moreover, in view of the large number and also the wording of the claims presently on file, which renders it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

In addition, the obscure definition of claims 32, 33, 35, 36, 70, 71, 76, 78 and 79, relating to an unidentified SEQ ID, and referring to the examples renders as well the search of these claims impracticable.

Consequently, the search has been carried out for those parts of the application which do appear to be clear, namely the particular sequences SEQ ID NOs.: 1, 2, 3, 4, 5, 6, and 8, variants, and uses thereof

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-31, 34, 37-69, 72-75, 77, 80-129, 136-140 and partially 141

Proteases capable of cleaving the beta secretase cleavage site of APP, variants thereof; polynucleotides encoding them; vectors and host cells comprising the same; antibodies for the polypeptides and uses of the foregoing in screening tests.

2. Claims: 130-135 and partially 141

APP isoform wherein the last two carboxy terminus amino acids are lysine residues.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/20881

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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